THE REGULATION OF INFLAMMATORY MEDIATORS IN HUMAN ENDOMETRIUM

Anne Elizabeth King

PhD
The University of Edinburgh
2000

Abstract

Menstruation and implantation are characterized by increased inflammatory mediator expression, leukocyte infiltration and stromal oedema. While the sex steroids, oestrogen and progesterone, are crucial to the control of endometrial function the molecular mechanisms involved remain unclear. The NFκB pathway is involved in the regulation of genes associated with the inflammatory and immune response. Progesterone has been found to inhibit this pathway by several mechanisms but there is currently little data regarding the role of the NFκB pathway in human endometrium. The aims of this research project have been to investigate the NFκB pathway, and mediators that interact with it, in endometrium. Additionally, progesterone control of the pathway intermediates has been studied.

NFκB pathway intermediates were identified in endometrium and decidua. The mRNA expression profiles of the inhibitory protein, IκBα, and an upstream kinase, TANK binding kinase 1, suggest that the pathway is activated during menstruation. Additionally, intermediates involved in the NFκB activating pathway are differentially regulated in first trimester decidua. The pathway that mediates proinflammatory signalling (MEKK1-IKKβ) to NFκB is downregulated in decidua consistent with the local immunosuppression that occurs during pregnancy. In contrast, intermediates involved in morphogenic signalling (NIK-IKKα) to NFκB are increased. This suggests a role in the expression of molecules crucial to successful pregnancy. The T47D cell line expresses high levels of progesterone receptor and was used as a cell model to study the effects of progesterone on the NFκB pathway. IκBα mRNA expression was found to be increased by progesterone while other pathway intermediates were unaffected by progesterone over the timecourse investigated.

CD40 is a proinflammatory signalling molecule that activates NFκB. CD40 was detected in the perivascular region of endometrium. Previously, it has been reported that chemokine expression is upregulated in this region premenstrually and the detection of CD40 in this area suggests a role in the control of inflammatory mediator

expression during menstruation. Messenger RNA expression for CD40 and its ligand, CD40L, was increased in decidua suggesting a role similar to that of NIK-IKKα. Secretory leukocyte protease inhibitor (SLPI) is an anti-inflammatory and antimicrobial molecule that also has inhibitory effects on the NFκB pathway. SLPI was localized to the glandular epithelium of endometrium from the mid-late secretory phase and was also detected in first trimester decidua. SLPI may provide antimicrobial protection at the time of implantation and during pregnancy. The detection of NFκB pathway intermediates, the CD40-CD40L system and SLPI in human endometrium supports a role for interacting control mechanisms in the regulation of inflammatory mediators in the uterus.

Contents

Abstract 1

Declaration 5

Acknowledgements 6

Abbreviations 7

Chapter 1: Literature Review

1.1 The Endometrium and Menstrual Cycle 11

1.1.1 The Ovarian Cycle 11

1.1.2 The Proliferative Phase 15

1.1.3 The Secretory Phase 15

1.1.4 Mechanisms of Menstruation 16

1.1.5 Decidualization and Implantation 20

1.2 Inflammatory events in endometrium 23

1.2.1 Leukocytes in endometrium 23

1.2.2 Inflammatory mediator expression in endometrium 26

1.3 Regulation of inflammatory mediators in endometrium 38

1.3.1 Oestrogen and progesterone receptor expression in endometrium 39

1.3.2 Nuclear factor kappa B (NFκB) 41

1.3.3 The NFκB signal transduction pathway – The IKK complex 44

1.3.4 The NFκB signal transduction pathway Upstream of the IKK complex 51

1.3.5 Activators of NFκB 53

1.3.6 Inhibitors of NFκB 60

1.4 Hypothesis and Aims 65

Chapter 2: General Methods

2.1 Human uterine tissue collection 70

2.2 RNA extraction and reverse transcription-polymerase chain reaction 73

2.3 Immunohistochemistry 86

2.4 *In vitro* culture 91

2.5 Enzyme linked immunosorbent assay (ELISA) 92

2.6 Statistical analysis 93

Chapter 3: The NFκB pathway in human endometrium and first trimester decidua

3.1 Introduction 95

3.2 Methods 97

3.3 Results 104

3.4 Discussion 123

Chapter 4: The CD40-CD40 ligand system in human endometrium and first trimester decidua

4.1 Introduction 138

4.2 Methods 140

4.3 Results 144

4.4 Discussion 163

Chapter 5: Secretory leukocyte protease inhibitor expression in human endometrium and first trimester decidua

5.1 Introduction 172

5.2 Methods 174

5.3 Results 179

5.4 Discussion 195

Chapter 6: Progesterone control of the NFκB pathway in the T47D cell line

6.1 Introduction 205

6.2 Methods 207

6.3 Results 210

6.4 Discussion 218

Chapter 7: General Discussion

7.1 Synopsis of results 222

7.2 Regulation of inflammatory mediators during menstruation 225

7.3 Regulation of inflammatory mediators in early pregnancy 229

7.4 Future studies 232

Bibliography 234

Appendices 1-4 281

Declaration

Except where due acknowledgement is made by reference the studies undertaken in this thesis were the unaided work of the author. No part of this work has been previously accepted for, or is currently being submitted in candidature for another degree.

Acknowledgements

I would like to acknowledge the support and encouragement of my supervisors, Professors Rodney Kelly and Hilary Critchley. In particular, I would like to thank them for their generosity with both their time and enthusiasm. I would also like to thank all members of their research groups, past and present – Gail Baldie, Fiona Denison, Teresa Drudy, Vivien Grant and Stuart Milne – for their friendly support and advice. Similarly, I would like to acknowledge the involvement of Professor Rick Phipps in the CD40 studies.

My project has relied heavily on the collection of human biopsies and I extend my thanks to Professor Hilary Critchley, Sister Joan Crieger and all theatre staff (Wards 34&35, Royal Infirmary of Edinburgh) involved in this. I would also like to thank Dr A.R.W Williams for histological dating of endometrial biopsies and Ian Swanston and Caroline Mutch for performing oestradiol and progesterone radioimmunoassays on serum samples.

I would like to acknowledge the graphical assistance of Ted Pinner and Tom McFetters, the technical advice of Sheila McPherson and the support of everyone in the Centre for Reproductive Biology. My PhD has been funded by the Medical Research Council.

Finally, I would like to thank all my family and friends for their support during my studies.

Abbreviations

ABC	avidin biotin peroxidase detection system
ANOVA	analysis of variance
AP-1	activator protein-1
cAMP	adenosine-3',5'-cyclic monophosphate
CD	cluster determinant
CD40L	CD40 ligand
cDNA	complementary DNA
COX-1/2	cyclo-oxygenase-1/2
E_2	oestradiol
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme linked immunosorbent assay
ER	oestrogen receptor
ERK	extracellular signal regulated kinase
$ET_{A/B}$	endothelin receptor A/B
FAM	6-carboxyflourescein
FSH	follicle stimulating hormone
GM-CSF	granulocyte/macrophage-colony stimulating factor
GR	glucocorticoid receptor
GRE	glucocorticoid response element
GRO$\alpha/\beta/\gamma$	growth related oncogene $\alpha/\beta/\gamma$
ICAM-1	intercellular adhesion molecule-1
IFNγ	interferon γ
IgG	immunoglobulin G
IKAP	IKK complex associated protein
IKK$\alpha/\beta/\gamma$	IκB kinase $\alpha/\beta/\gamma$
IL-1/6 etc	interleukin-1/6 etc
IL-1ra	IL-1 receptor antagonist
IL-1Rt I/II	IL-1 receptor type I/II
iNOS	inducible nitric oxide synthase

IRAK	IL-1 receptor associated kinase
IRF-1	interferon regulatory factor-1
IκB	inhibitor of NFκB
JAK	Janus kinase
JOE	2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein
LGL	large granular lymphocytes
LH	luteinizing hormone
LIF	leukaemia inhibitory factor
LMP	last menstrual period
LNG-IUS	levonorgestrel intrauterine system
LPS	lipopolysaccharide
LTA	lipoteichoic acid
MCP-1	monocyte chemoattractant protein-1
MEKK1/2/3	mitogen activated protein kinase/ERK kinase kinase 1/2/3
MIP1α/β	macrophage inflammatory protein 1 α/β
MMP	matrix metalloproteinase
NBF	neutral buffered formalin
NCoR	nuclear receptor corepressor
NEMO	NFκB essential modulator
NFκB	nuclear factor kappa B
NIK	NFκB inducing kinase
NK	natural killer
P_4	progesterone
PBS	phosphate buffered saline
PG	prostaglandin
PGDH	prostaglandin dehydrogenase
$PGE_2/F_{2\alpha}$	prostaglandin $E_2/F_{2\alpha}$
PLSD	protected least squares difference
PMA	phorbol,1-myristate,13-acetate
PR	progesterone receptor
PRE	progesterone response element
RANTES	regulated upon activation, normal T cell expressed

RT-PCR	reverse transcription-polymerase chain reaction
SLPI	secretory leukocyte protease inhibitor
SMRT	silencing mediator for retinoid and thyroid hormone receptor
STAT	signal transducer and activator of transcription
TAK1	transforming growth factor β activated kinase 1
TAMRA	6-carboxytetramethylrhodamine
TANK	TRAF family member associated NFκB activator
TBK1	TANK binding kinase-1
TGFα/β	transforming growth factor α/β
Th1/2	T helper 1/2
TIMP	tissue inhibitor of MMP
TNFR	tumour necrosis factor receptor
TNFα	tumour necrosis factor α
TRAF	TNF receptor associated factor
VCAM-1	vascular cell adhesion molecule-1

1. Literature Review

1.1 The Endometrium and Menstrual Cycle

The human endometrium is a dynamic tissue capable of supporting the implantation and development of an embryo. In order to provide this support the tissue undergoes a series of cyclical changes which prepare it for potential implantation. In the absence of pregnancy the endometrium is shed during menstruation and then regenerates during the next menstrual cycle. The endometrium consists of two layers, the basalis and the functionalis. Morphological changes during the menstrual cycle only occur in the functionalis and it is this layer which is shed during menstruation. Regeneration of the endometrium occurs from the basalis after menstruation. This sequential shedding, regeneration and differentiation is driven by the ovarian sex steroids.

1.1.1 The Ovarian Cycle

Cyclical ovarian follicle development and hence, sex steroid production, occurs under the control of the hypothalamic hormone, gonadotrophin releasing hormone, and the pituitary gonadotrophins, follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Johnson and Everitt, 2000). At the beginning of each cycle there is relaxation of inhibitory mechanisms allowing the increased secretion of FSH from the pituitary. Under the influence of FSH a cohort of antral follicles begins to undergo preovulatory development. One of the follicles will become dominant and will eventually ovulate. This follicle begins to secrete oestrogens exerting an inhibitory effect on the pituitary. As a result, FSH secretion decreases to a level that will not support the growth of the other follicles. As the dominant follicle further develops, oestrogen levels continue to rise. Once a threshold level is reached positive feedback on the pituitary occurs resulting in the LH surge which is responsible for ovulation. Under the control of LH the theca and granulosa cells of the follicle luteinize forming the corpus luteum. It is the corpus luteum that is responsible for the high levels of oestrogen and particularly, progesterone, in the luteal phase of the cycle. Negative feedback upon the pituitary is maintained by these high concentrations of sex steroids. In the absence of conception and embryonic signals,

the corpus luteum degenerates and sex steroid production decreases. This relaxes the negative inhibition allowing FSH secretion to increase and the cycle to recommence.

This cyclical production of oestrogen and then, progesterone, (Figure 1) by the ovarian follicle and corpus luteum is reflected by changes to the morphology of the endometrium (Johannisson, et al., 1982). These endometrial changes have now been well documented: firstly, by Noyes *et al* (1950) and more recently by others (Buckley and Fox, 1989; Strauss III and Gurpide, 1991). It should also be noted that the leukocyte populations in endometrium undergo cyclical changes and have a role in endometrial function (King, et al., 1998; Loke and King, 1995). This is discussed in detail in section 1.2.1.

Figure 1: Concentrations of sex steroids and pituitary gonadotrophins in the circulation during the menstrual cycle **(a).** Oestradiol (E2) and progesterone (P4). **(b).** Follicle stimulating hormone (FSH) and luteinizing hormone (LH). Data adapted from Marshall and Odell, 1989.

Figure 1a

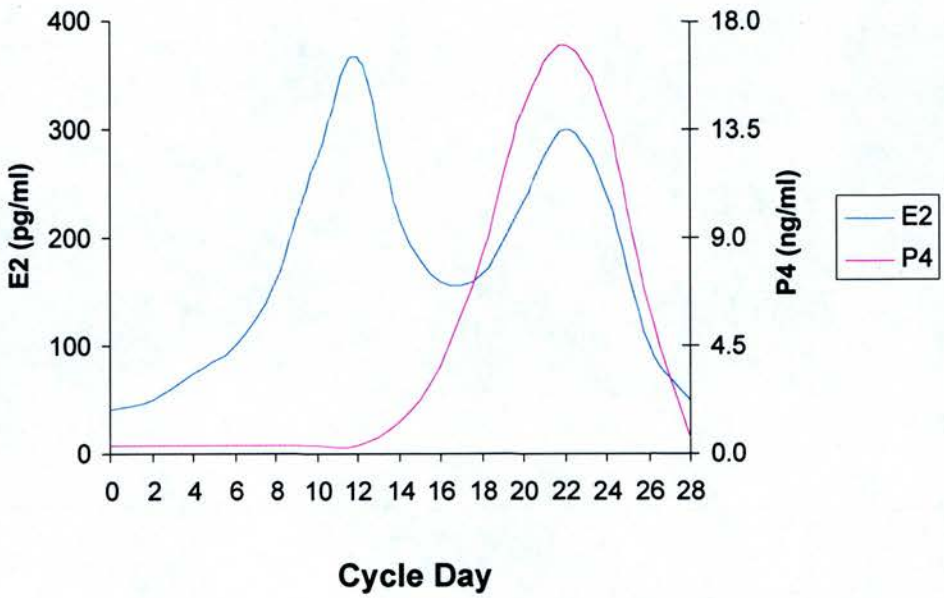
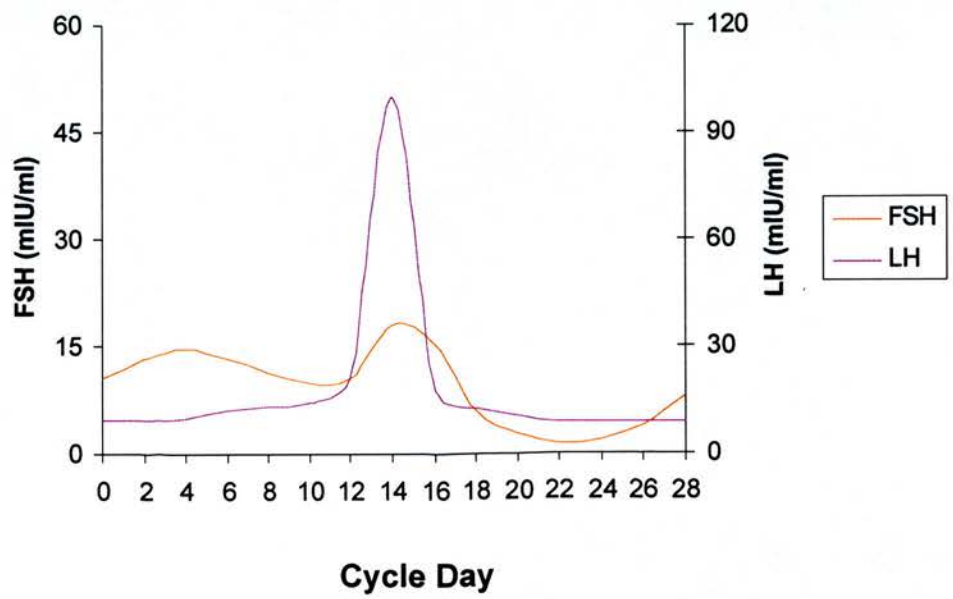


Figure 1b



1.1.2 The Proliferative Phase

The proliferative phase of the menstrual cycle occurs under the influence of increasing oestradiol levels from the developing follicle. The early proliferative phase lasts from approximately day 4 to day 7 of a 28 day cycle and is characterized by the regeneration of the endometrium after menstruation. The glands are narrow and straight, the surface epithelium is thin and the stroma compact. During the mid and late proliferative phase (day 8 to day 10; day 11 to day 13) there is continued growth of glands and stroma with the glands becoming tortuous as ovulation approaches.

1.1.3 The Secretory Phase

After ovulation, under the influence of progesterone, proliferation ceases and there is differentiation of the endometrium. The early secretory phase lasts from ovulation until day 18. The first sign that ovulation has occurred is the appearance of subnuclear vacuoles in the glandular epithelium (day 16). These vacuoles push the nuclei to the centre of the cells resulting in the alignment of nuclei. This is accompanied by increased tortuousity of the glands. In the mid secretory phase (day 19 to day 23) the secretory activity of the glands becomes apparent with maximal secretion around day 20 and stromal oedema peaks around day 22/23. In the late secretory phase (day 24 to day 28) there is differentiation of spiral arterioles. Perivascular stromal cells become more apparent as their cytoplasm increases in volume and their nuclei enlarge. These are predecidual cells and on day 25 predecidual change also begins under the surface epithelium forming a solid sheet of tissue. This decidual change will occur throughout the endometrium in the event of pregnancy. If pregnancy does not occur, glandular secretion becomes exhausted and the glands have a saw-toothed appearance. Growth of the endometrium ceases and dehydration results in shrinkage. Infiltration of leukocytes begins to occur around day 26. Focal necrosis becomes apparent and menstruation begins (day 1).

1.1.4 Mechanisms of menstruation

Menstruation occurs when the demise of the corpus luteum causes circulating levels of oestradiol and progesterone to fall and involves the sloughing of the superficial layer of the endometrium (Cameron, 1992). This occurs haphazardly: some areas are shed while others are being repaired. Menstruation occurs only in humans, Old World monkeys, anthropoid apes and two non-primate species, the elephant shrew (*Elephantus myuras jamesoni*) and some bats (e.g. *Glossophaga sorcinina*). It is believed that menstruation is necessary as a result of stromal changes (e.g. decidual cell reaction, stromal oedema, vascular development, leukocyte infiltration) which, in menstruating primates, occur in the secretory phase of the cycle, under the influence of progesterone and regardless of whether a blastocyst is present. This may provide a maternal protective mechanism which counteracts the aggressive implantation which occurs in these species (Finn, 1987; Finn, 1996). Due to the terminal differentiation of the stroma during the menstrual cycle menstruation must occur, in the absence of pregnancy, in order to allow regeneration of the endometrium. Menstruation does not occur in mammals with less aggressive implantation. For example, in rodents the trophoblast adheres to the luminal epithelium but does not invade the endometrium. The trophoblast phagocytoses the epithelial cells leaving the blastocyst inside the endometrial stroma. Once this has happened the blastocyst becomes invasive and stromal changes occur. It is thus unnecessary for these mammals to menstruate as the stromal reaction occurs only in the presence of pregnancy. However, when a hormone primed mouse uterus is stimulated with a drop of oil (mimicking the trophoblast) and the progesterone source withdrawn, a menstruation-like response occurs (Finn and Pope, 1984).

The mechanisms currently considered to be involved in menstruation were first described by Markee (1940) in his experiments on the rhesus monkey. These experiments involved the intraocular transplantation of endometrium allowing it to be observed during menstruation. It was found that prior to menstruation regression of the endometrium occurred. Striking changes were also noted in the endometrial blood vessels. The arteries that supply the endometrium are straight when in the

basalis region but become coiled when they enter the functionalis. As the secretory phase of the menstrual cycle progressed the spiral arteries became increasingly coiled until circulation slowed and stasis occurred due to the increased resistance encountered in the vessels. About 4 to 24 hours premenstrually, vasoconstriction of the spiral arteries was found to occur. This caused the functionalis to become anoxic and resulted in bleeding. Leukocyte infiltration accompanied menstruation. Vasoconstriction of the basal part of the arteries then occurred terminating bleeding.

The vascular changes found to be associated with menstruation have led to current theories that vasoactive substances present in the endometrium are likely to be involved in the menstrual process. Prostaglandins (PGs) are lipid mediators that are produced from arachidonic acid via the cyclo-oxygenase enzymes, COX-1 and COX-2. Lipid substances were first discovered in the menstrual fluid by Pickles (Pickles, 1957) and there is now much evidence suggesting a role for PGs in menstruation (Baird, et al., 1996). First, PGE_2, a vasodilator, and $PGF_{2\alpha}$, a vasoconstrictor, have been detected in the menstrual fluid. The vasoconstrictive actions of $PGF_{2\alpha}$ imply a role in the vascular events that precede menstruation. The ratio of PGE_2: $PGF_{2\alpha}$ is found to be increased in the menstrual fluid of women suffering from menorrhagia (Cameron, et al., 1987; Rees, et al., 1984) and administration of COX inhibitors reduces blood loss suggesting that aberrant expression of the vasodilator PGE_2 causes increased bleeding (Fraser, 1992). Second, *in vitro* culture studies suggest that in the secretory phase of the menstrual cycle the endometrium has a greater capacity to synthesize PGs although progesterone inhibits actual release (Abel and Baird, 1980). As a result, when progesterone is withdrawn (i.e. premenstrually) endometrial prostaglandin production reaches maximal levels. Finally, the prostaglandin synthesizing and metabolizing enzymes (COX-2 and prostaglandin dehydrogenase; PGDH) have been found to be under hormonal control. Progesterone stimulates the activity of PGDH thus increasing prostaglandin metabolism and activity is high in the secretory phase (Casey, et al., 1980). Recently, progesterone has been found to upregulate PGDH expression at the transcriptional level (Greenland, et al., 2000). This is consistent with the culture data described above. High levels of PGDH are usual in first trimester decidua. However, when RU486 (an anti-progestogen) is

administered to women PGDH expression falls, particularly around blood vessels (Cheng, et al., 1993b). Expression of COX-2, while present in the glandular epithelium throughout the menstrual cycle, is increased in the perivascular region upon premenstrual progesterone withdrawal (Critchley, et al., 1999; Jones, et al., 1997). While the epithelium is the major site of prostaglandin production in endometrium (Lumsden, et al., 1984) it is the synthesis of prostaglandins in close proximity to blood vessels that is likely to be most relevant to menstruation. A simultaneous increase in COX-2 and decrease in PGDH expression upon progesterone withdrawal is likely to result in increased PG production in the proximity of the blood vessels.

More recently, studies on the role of vasoactive mediators in menstruation have focused on a group of 21 amino acid peptides, the endothelins (Marsh, et al., 1996). They are cleaved from precursor (pro- and preproendothelins) proteins and to date, three endothelins have been identified, endothelin-1 (the most potent vasoconstrictor known), 2 and 3. The endothelins act via two G-protein coupled receptors, ET_A and ET_B. Endothelin-1, 2 and 3 mRNA (O'Reilly, et al., 1992) and protein (Cameron, et al., 1993) have been detected throughout the menstrual cycle in human endometrium. The proteins have been reported to be localized to the glandular and luminal epithelium (Cameron, et al., 1993; Salamonsen, et al., 1992) with endothelin-1 also present in the stroma and in some endothelial cells (Ohbuchi, et al., 1995; Salamonsen, et al., 1992). Endothelin-1 protein (Ohbuchi, et al., 1995) and mRNA (Economos, et al., 1992) levels have been found to increase in the premenstrual-menstrual phase suggesting a role in menstruation. Also, the mRNA (Kubota, et al., 1995; O'Reilly, et al., 1992) and protein (Collett, et al., 1996) for ET_A and ET_B have been detected in endometrial samples. These studies have reported that the ratio of ET_A:ET_B varies throughout the menstrual cycle with an increase in the expression of ET_B in the secretory and menstrual phases. ET_A has been localized to the endometrial stroma with maximal expression during the proliferative phase. ET_B immunoreactivity was found to increase in the glandular epithelium of the basalis and the functionalis regions in the early secretory and late secretory phases, respectively. The receptor was localized to both the epithelium and the stroma in the menstrual

phase (Collett, et al., 1996). It has been suggested that the endothelins may act via the ET_B receptor to cause vasoconstriction during menstruation.

An alternative theory explaining the events surrounding menstruation involves the production of matrix metalloproteinases (MMPs) (Salamonsen, 1998; Salamonsen and Woolley, 1999). There are several major subgroups of MMPs: the collagenases (e.g. MMP-1), the gelatinases (MMP-2 and 9), the stromelysins (MMP-3, 10 and 11) and the membrane-type MMPs. MMPs are secreted as inactive zymogens, which are activated by proteases, and together, the MMPs are capable of degrading the extracellular matrix. Tissue inhibitors of MMPs (TIMPs) are endogenous inhibitors that act by forming 1:1 complexes with the MMP. The suggestion that MMPs are involved in menstruation resulted from the observation that small lesions are present in the luminal epithelium of endometrium prior to any blood loss. This is followed by rapid degeneration of the functionalis region resulting in the exposure of open blood vessels (Ludwig and Spornitz, 1991). Also, the basal lamina, which supports decidualized cells and endothelium, degenerates prior to bleeding (Roberts, et al., 1992). Expression of MMPs is consistent with a role in menstruation with increased expression of proMMP-1 and proMMP-3 mRNA in the perimenstrual phase (Hampton and Salamonsen, 1994). Another study reported similar upregulation of mRNA for several MMPs (particularly MMP-1, MMP-3, MMP-9 and MMP-10) during menstruation while TIMP-1 mRNA expression remained constant throughout the menstrual cycle (Rodgers, et al., 1994). Additionally, progesterone withdrawal has been found to increase MMP release in *in vitro* culture of endometrial stromal cells (Lockwood, et al., 1998; Salamonsen, et al., 1997). No effect of progesterone withdrawal was found on TIMP release suggesting that an increased ratio of MMP:TIMP may be involved in menstruation (Salamonsen, et al., 1997). Increased expression of MMPs as a result of progesterone withdrawal has also been reported in a non-human primate model of menstruation (Brenner, et al., 1996). This study suggested that *in vivo* progesterone withdrawal acts indirectly, via locally produced cytokines, to upregulate MMPs. Various mediators such as tumour necrosis factor α (TNFα), interleukin-1 (IL-1) and prostaglandins have been suggested as potential regulators of MMP expression. The infiltrating lymphoid cells which are present

prior to menstruation have been suggested to be one source of these mediators (Salamonsen and Woolley, 1996). Although MMPs are clearly involved in the tissue breakdown involved in menstruation their activation is likely to be several steps downstream of the initial vasoconstriction events reported by Markee.

1.1.5 Decidualization and Implantation

Successful implantation requires the simultaneous development of the endometrium to a receptive state and the embryo to blastocyst stage. The endometrium is receptive to implantation only for a few days in each menstrual cycle (the implantation window). This time is between day 20 and 24 in the human (Harper, 1992). The implantation window falls in the mid-late secretory phase (section 1.1.3) and at this time the endometrium is characterized by the presence of pinopodes on the luminal epithelial cells. These are thought to withdraw fluid from the uterine lumen allowing close contact between the epithelial cells and the developing embryo (Martel, et al., 1989). Nuclear channel systems and giant mitochondria are also apparent in the glandular and luminal epithelium around this time. The function of these features is unclear but involvement in secretory events and energy provision has been suggested (Li, et al., 1994). As detailed in section 1.1.3, the transformation of endometrium to decidua begins in the secretory phase of the cycle under the influence of progesterone. This differentiation of the endometrium is critical to the establishment of pregnancy and there are characteristic changes in all compartments of the endometrium (King, 2000; Loke and King, 1995). First, secretion from the glandular epithelium is maximal during the implantation window with production of various hormonally regulated proteins including progesterone-associated endometrial protein (also known as glycodelin, placental protein 14, lactoglobulin, α-2 microglobulin, pregnancy-associated α-2 globulin, α uterine protein) and albumin. In decidua, the glands in the upper two thirds of the tissue become atrophied and are non-secretory while those in the lower third continue secretory activity. Second, as detailed above, the stroma also shows predecidual change that develops fully in the event of implantation. This stromal differentiation begins around the spiral arterioles and beneath the surface epithelium with the cells becoming plump and glycogen rich.

Also, extracellular matrix (ECM) proteins are distributed around each stromal cell with the decidual ECM being particularly rich in laminin and fibronectin. Stromal cells are responsible for the secretion of α1 pregnancy-associated endometrial protein (also known as placental protein 12, insulin-like growth factor binding protein-1) and prolactin. The precise roles of these proteins, and those secreted by the glandular epithelium, is unclear although immunosuppressive activities, regulation of trophoblast growth and fetal effects have been suggested (Strauss III and Gurpide, 1991). Finally, increased coiling of the spiral arterioles begins in the secretory phase and continues in decidua. Large granular lymphocytes are the main leukocyte population in first trimester decidua and these are described in section 1.2.1. The functions of decidua are not fully understood although it is thought that it may have a role in controlling the invasion of trophoblast into the maternal tissue and also, in regulating immune events during pregnancy.

After fertilization the zygote undergoes successive cleavage events forming a morula which enters the uterine lumen around 72-96 hours post fertilization. The blastocyst forms on day 5 and comprises an inner cell mass with an outer layer of trophectoderm. Hatching from the zona pellucida occurs around 1-2 days prior to penetration of the endometrium and at this time, the peripheral cytotrophoblast differentiates into syncytiotrophoblast. Human chorionic gonadotrophin begins to be produced by the embryo preventing the demise of the corpus luteum.

Implantation occurs in three stages: apposition, adhesion and invasion. The apposition stage occurs when the hatched blastocyst is present in the uterine lumen and in close, but not direct, contact with the luminal epithelium. Between days 6-7 postfertilization adhesion of the blastocyst to the uterine epithelium occurs. This involves direct interaction of the trophectoderm with the epithelium. After this attachment, the trophoblast invades the endometrium. The trophoblast cells penetrate through the surface epithelium into the uterine stroma. Some of the cytotrophoblast cells extend through the syncytiotrophoblast and invade the decidua. These cells fuse forming the cytotrophoblast shell which surrounds the inner surface of the implantation site and anchors the placenta to the decidua. The extravillous

trophoblasts also invade the decidual spiral arterioles and become incorporated into the vascular wall, changing its plasticity. This allows a continuous blood supply to the fetus and isolates it from the pulsatility of the maternal circulation. (Aplin, 1991; Edwards, 1995; Pijnenborg, et al., 1981; Tabibzadeh and Babaknia, 1995).

Implantation clearly involves a tightly orchestrated series of events which requires the controlled expression of a variety of molecules (Giudice, 1999; Simon, et al., 1996; Tabibzadeh and Babaknia, 1995). First, prior to attachment to the endometrial epithelium, signalling between the developing blastocyst and the endometrium is necessary. This is likely to occur via soluble mediators. For example, leukaemia inhibitory factor (LIF) is a cytokine that has been shown to be crucial for implantation in mice. LIF expression is maximal in mouse endometrium on the day of implantation (Bhatt, et al., 1991) and the LIF knockout mouse, while demonstrating normal ovulation and fertilization, had failed implantation (Stewart, et al., 1992). In humans, LIF expression is also found to be maximal during the implantation window with expression being primarily in the glandular epithelium at this time (Charnock-Jones, et al., 1994). The precise role of LIF in human implantation is unknown but involvement in blastocyst-endometrial communication is likely and effects on trophoblast differentiation have been demonstrated (Nachtigall, et al., 1996). The role of the IL-1 system in implantation will be discussed in section 1.2.2.

Integrins are likely to be involved in the adhesion phase of implantation promoting the formation of contacts between the endometrial epithelium and the trophectoderm. The integrins are heterodimeric, transmembrane glycoproteins that have an extracellular domain that binds extracellular matrix components (e.g. fibronectin, laminin) and an intracellular domain that interacts with cytoskeletal proteins. The integrins are composed of an α and β chain. The human endometrium, like other tissues, has been found to constitutively express some integrins but also displays several only during the implantation window (Lessey, et al., 1992). Expression of $\alpha_4\beta_1$, $\alpha_v\beta_3$ and $\alpha_1\beta_1$ has been reported to correlate with the time of implantation (Lessey, et al., 1994; Lessey, et al., 1992; Tabibzadeh, 1992). Indeed, aberrant

integrin expression may be involved in infertility. Lack of $\alpha_v\beta_3$ expression has been associated with both classical luteal phase defect (Lessey, et al., 1992) and unexplained infertility (Lessey, et al., 1995).

The invasion of the endometrium by trophoblasts also involves the expression of various integrins e.g. as cytotrophoblasts migrate through the endometrium there is a switch from $\alpha_6\beta_4$ to $\alpha_1\beta_1$ and $\alpha_5\beta_1$ expression (this means that as the cytotrophoblast moves into the decidua it has an increased capacity to bind to fibronectin, a major component of decidualized stroma) (Burrows, et al., 1993; Damsky, et al., 1992). In addition to this, trophoblasts secrete MMPs and serine proteases that are involved in the tissue degradation that occurs during implantation. The invasive human trophoblast has been found to secrete MMP9 and MMP2 (Shimonovitz, et al., 1994) during the first trimester. Both of these MMPs degrade collagenase IV which is the main component of the uterine basal membrane. Human decidua produces TIMPs, in particular TIMP3, which are involved in the limitation of MMP action (as described in section 1.1.4) and therefore, regulation of trophoblast invasiveness (Higuchi, et al., 1995).

1.2 Inflammatory events in endometrium

Both implantation and menstruation are associated with inflammatory-like events (Finn, 1986; Kelly, 1994). Leukocyte infiltration of the endometrium, increased inflammatory mediator expression and oedema have been reported to occur during these processes.

1.2.1 Leukocytes in endometrium

Leukocytes are present in endometrium throughout the menstrual cycle constituting approximately 8.2% of the stromal cells in the proliferative phase with this figure rising to 31.7% in first trimester decidua (Bulmer, et al., 1991). Lymphoid aggregates are present in the basalis region of endometrium throughout the menstrual cycle and contain T cells and B cells surrounded by a halo of macrophages. T cells

are also found scattered in the endometrial stroma and in an intraepithelial location while B cells are rarely found outwith lymphoid aggregates. Macrophages constitute about 20% of the leukocytes present in endometrium and are present throughout the cycle with a small increase premenstrually. A scavenger role during menstruation has been suggested. In decidua macrophages are found at the implantation site often in contact with extravillous trophoblast (Bulmer, et al., 1988; Loke and King, 1995).

Large granulated lymphocytes (LGL; endometrial natural killer (NK) cells) are also present in endometrium. These cells differ from classical NK cells in that they are $CD56^{++}CD16^{-}$ whereas classical NK cells are $CD56^{-}CD16^{+}$. The LGLs are present in low numbers in the proliferative phase and increase in the mid secretory phase with numbers peaking in the late secretory phase (i.e. they are present during the implantation window). In pregnancy the cells constitute approximately 70% of the leukocytes in first trimester decidua and are present until the 2^{nd} trimester with numbers subsequently declining (Loke and King, 1995). Proliferation of the LGLs within the endometrium has been shown by investigation of the proliferation marker, Ki67 (Kammerer, et al., 1999), and migration from the circulation is also possible (Marzusch, et al., 1993). Progesterone is crucial for the appearance of these cells as in the absence of pregnancy the cells undergo apoptosis a few days prior to menstruation. The function of uterine LGLs is unclear but may involve a role in implantation and placentation (King, 2000; King, et al., 1998; Loke and King, 1995).

Prior to menstruation there is an influx of eosinophils (Jeziorska, et al., 1995) and neutrophils (Poropatich, et al., 1987) into the endometrium and these cells are likely to be involved in the tissue breakdown involved in menstruation. Figure 2 details the leukocyte populations present in endometrium throughout the menstrual cycle.

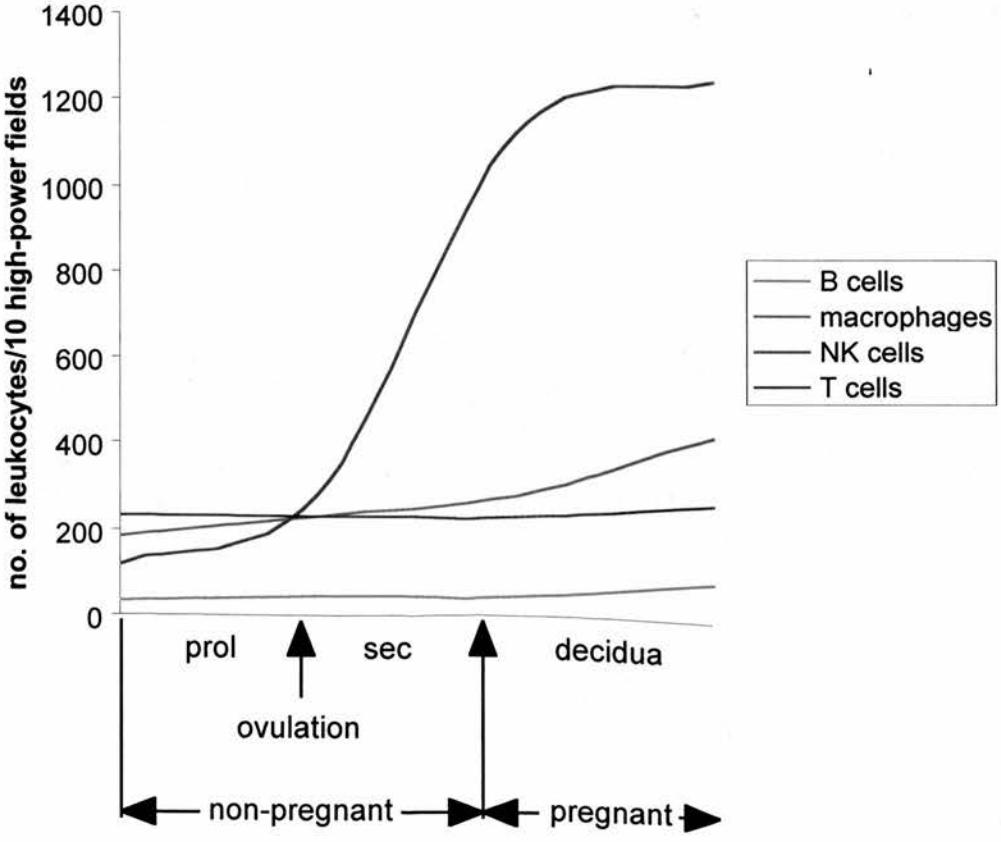


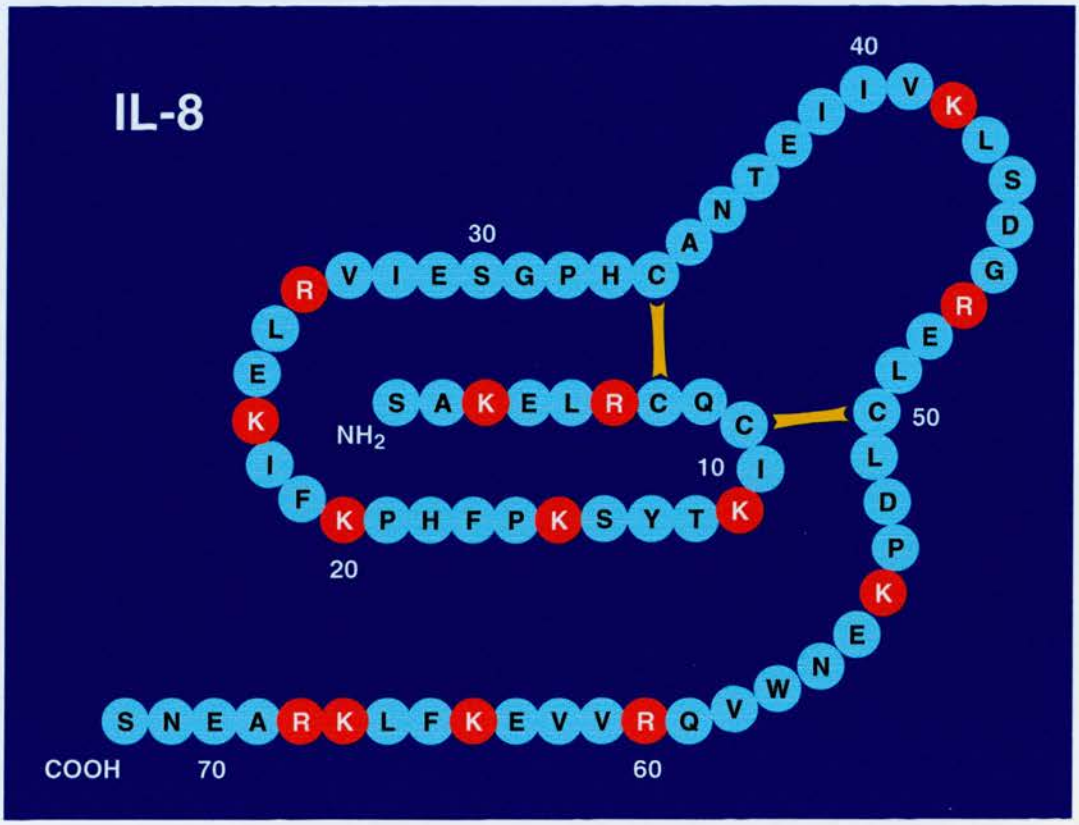
Figure 2: Leukocyte populations in human endometrium (prol = proliferative phase; sec = secretory phase) and first trimester decidua. Data and illustration adapted from Loke and King, 1997.

Although progesterone is crucial to the presence of LGLs in the endometrium and progesterone withdrawal is involved in the infiltration of neutrophils and eosinophils premenstrually it is likely that these effects are indirect as progesterone receptors have not been detected in these cells (King, et al., 1996). Instead it is likely that the effects of progesterone and its subsequent withdrawal are mediated via locally produced molecules.

1.2.2 Inflammatory mediator expression in endometrium

Chemokines are cytokines which have chemotactic activity. They have four cysteines and two disulphide bonds (Figure 3) and are divided into two main groups dependent on the position of their first two cysteines. CXC chemokines (α-chemokines; first two cysteines separated by one amino acid) are chemoattractant mainly for neutrophils although those that do not contain an N terminal glutamic acid-leucine-arginine motif attract lymphocytes. In contrast, the CC chemokines (β-chemokines) attract monocytes, basophils, eosinophils and T-lymphocytes. Interleukin-8 (IL-8), growth-related oncogene α (GROα), GROβ and GROγ belong to the CXC group while monocyte chemoattractant protein 1 (MCP1), macrophage inflammatory protein 1α and 1β (MIP1α/β) and regulated upon activation, normal T cell expressed and secreted (RANTES) are representative of the CC chemokines (Luster, 1998; Rollins, 1997). Chemokines act via 7 transmembrane spanning G-protein coupled receptors (Murphy, 1996).

Figure 3: The structure of the CXC chemokine, interleukin-8. Basic amino acids are shown in red.


IL-8
40
30
50
10
20
60
70
NH_2
COOH
S A K E L R C Q C I
K P H F P K S Y T K
R V I E S G P H C A N T E I I V K L S D G R E L C L D P K E N W V Q R V V E K L F K R K A E N S

IL-8, a representative CXC chemokine, is a potent neutrophil chemoattractant and activator (Yoshimura, et al., 1987). It is produced by several cell types including monocytes (Walz, et al., 1987), T lymphocytes (Gregory, et al., 1988), fibroblasts (Larsen, et al., 1989b), endothelial cells (Strieter, et al., 1988) and epithelial cells (Elner, et al., 1990). In addition to a neutrophil chemotactic role IL-8 has been implicated in T cell chemotaxis (Larsen, et al., 1989a), angiogenesis (Koch, et al., 1992) and mitogenesis (Tuschil, et al., 1992; Yue, et al., 1994).

In a reproductive context IL-8 has been found to be produced by cultured endometrial epithelial and stromal cells (Arici, et al., 1993), amniotic fluid (Laham, et al., 1993) and choriodecidual cells (Kelly, et al., 1992). It is believed to be involved in the mechanism of cervical ripening. IL-8 production is found to increase in the cervix at the time of ripening (Kelly, et al., 1992; Sennstrom, et al., 1997) and, in synergy with PGE_2, is believed to be responsible for the recruitment of neutrophils (Colditz, 1990; Rampart, et al., 1989). The entry of neutrophils into the cervix is thought to have a role in the breakdown of collagen (via neutrophil collagenase) causing cervical softening (Junqueira, et al., 1980). In endometrium a similar synergistic action between IL-8 and PGE_2 has been suggested. IL-8 has been reported to be present in the endometrial glandular epithelium (Arici, et al., 1998) and in a perivascular location (Critchley, et al., 1994; Jones, et al., 1997) although, more recently, the mRNA has been localized mainly to the perivascular area (Milne, et al., 1999). Progesterone control of IL-8 in endometrium is likely as culture studies have shown that progesterone decreases IL-8 secretion by endometrial explants (Kelly, et al., 1994). This is consistent with reports of increased expression of perivascular IL-8 premenstrually (Jones, et al., 1997) and in a progesterone withdrawal model (Critchley, et al., 1999). The expression of COX-2 is also found to increase perivascularly in the premenstrual phase (Jones, et al., 1997) and the expression of these molecules is coincident with the influx of neutrophils reported prior to menstruation.

MCP-1, a CC chemokine, is produced by several cell types including fibroblasts (Yoshimura and Leonard, 1990), endothelial cells (Sica, et al., 1990), smooth muscle

cells (Cushing, et al., 1990) and monocytes (Yoshimura, et al., 1989). It is chemotactic mainly for monocytes (Rollins, 1991) but is also involved in T cell (Carr, et al., 1994) and NK cell (Allavena, et al., 1994) chemotaxis and in basophil activation (Bischoff, et al., 1992). Similar to IL-8, MCP-1 has been found to be secreted by cultured endometrial epithelial and stromal cells (Arici, et al., 1995) and is coexpressed with IL-8 and COX-2 in the endometrial perivascular cells (Jones, et al., 1997). MCP-1 is also thought to be involved in premenstrual leukocyte recruitment as expression increases in the perivascular region upon progesterone withdrawal (Critchley, et al., 1999; Jones, et al., 1997). This is in keeping with reports of progesterone mediated suppression of MCP-1 secretion in human endometrial stromal cells (Arici, et al., 1999), choriodecidual cells and the T47D breast cancer cell line (Kelly, et al., 1997).

In addition to the chemokines, many other cytokines have been shown to be important in endometrial physiology (Tabibzadeh, 1991). The roles of the highly pleiotropic cytokines, TNFα and IL-1, will be discussed with further descriptions of inflammatory molecule expression in human endometrium shown in Table 1.

TNFα has wide ranging actions including control of the inflammatory response (Old, 1985), monocyte chemotaxis (Ming, et al., 1987) and angiogenic effects (Frater-Schroder, et al., 1987). The expression of TNFα in endometrium has been described by several groups. While it is clear that the endometrial glandular epithelium is the major site of expression, the regulation of TNFα throughout the menstrual cycle is controversial. First, Hunt et al (1992) reported both epithelial and stromal expression of TNFα to increase during the proliferative phase, decrease in the early secretory phase and then increase in the mid-late secretory phase. In contrast, Philippeaux et al (1993) found weak expression in the glandular epithelium and arteries in the proliferative phase with strong expression in the secretory phase. Tabibzadeh (1995b) described increasing TNFα production throughout the cycle with a peak during menstruation. Although there are differences between these results they have all described high expression of TNFα in the late secretory phase. However, a recent study has suggested that TNFα expression is low at the beginning and end of the

menstrual cycle with maximal expression in the mid-late proliferative and early-mid secretory phases (Tabibzadeh, et al., 1999). Cell culture experiments have also produced conflicting findings. It has been suggested that TNFα release from epithelial cells derived from late proliferative endometrium is greater than from cells derived from other cycle stages (Laird, et al., 1996). However, a second study reported that TNFα release was maximal from epithelial cells derived from late secretory endometrium (Tabibzadeh, et al., 1999). Due to these disparate findings the role of TNFα in endometrium is also unclear. Regulation of DNA synthesis in the early proliferative phase (Hunt, 1993) and cell differentiation and tissue remodelling during implantation (Terranova, et al., 1995) have been suggested as possible functions. Tabibzadeh (1996; 1995b) has suggested that the reported increase in TNFα expression in the late secretory phase is consistent with a role in menstruation due to the ability of the cytokine to induce apoptosis and compromise vascular integrity. Steroid hormone control of TNFα expression has been described with progesterone suppression of TNFα production reported in activated mouse macrophages (Miller and Hunt, 1998) and in human endometrial epithelial cells derived from the secretory phase of the cycle (Laird, et al., 1996). Oestradiol withdrawal has also been found to upregulate TNFαmRNA expression in the epithelial cells of a human endometrial cancer grown in nude mice (Tabibzadeh, et al., 1999).

The IL-1 system is comprised of IL-1α and β, IL-1 receptor antagonist (IL-1ra) and the two IL-1 receptors, type I and II (IL-1RtI and IL-1RtII; only IL-1 RtI is functional). IL-1 was originally thought to be a product of macrophages but it is now clear that it is produced by many cell types including fibroblasts, endothelial cells and smooth muscle cells (Ibelgaufts, 1994). Both forms of the IL-1 receptor can bind IL-1α and β while IL-1ra binds only to IL-1RtI. The IL-1 system has been detected in human endometrium and is thought to have potentially important roles in implantation and menstruation. IL-1β has been detected in the endometrial endothelium, isolated stromal cells (Simon, et al., 1993a) and epithelium (Tabibzadeh and Sun, 1992) with expression reported to increase in the secretory phase of the cycle (Simon, et al., 1993a). IL-1RtI is expressed by endometrial epithelial cells,

particularly those of the luminal epithelium, with expression again increasing in the secretory phase (Simon, et al., 1993a; Simon, et al., 1993b)]. IL-1ra is also expressed by endometrial epithelial cells with highest expression in the luminal epithelium. In contrast to IL-1β and IL-1RtI, IL-1ra expression is decreased in the secretory phase (Simon, et al., 1995). The expression of both IL-1 and its receptor in the secretory phase suggests a role in the mechanisms involved in implantation. In support of this, IL-1 production by preimplantation embryos has been detected (Sheth, et al., 1991) and the IL-1 system is present in early implantation sites (Simon, et al., 1994a). Also, administration of human recombinant IL-1ra to mice has been used to block signal transduction via IL-1RtI. This was found to prevent attachment of embryos to endometrium (Simon, et al., 1994b). *In vitro* IL-1 has also been found to stimulate the production of other molecules involved in implantation such as LIF (Arici, et al., 1995), IL-6 (Laird, et al., 1994) and PGE_2 (Tabibzadeh, et al., 1990). In addition to a role in implantation, the wide-ranging ability of IL-1 to induce expression of other inflammatory mediators (e.g. IL-8, MCP-1, TNFα) along with stimulation of MMP production is consistent with a role in menstruation.

Table 1: Examples of inflammatory molecules present in human endometrium.

(A). Chemokines.

(B). Cytokines.

(C). Growth factors.

(D). Other inflammatory molecules.

A. Chemokines

Inflammatory mediator	Reported source in endometrium	Suggested function in endometrium
Chemokines		
Eotaxin	Perivascular cells Glandular & surface epithelium Eosinophils	Premenstrual eosinophil recruitment Epithelial expression suggests additional roles (Zhang, et al., 2000)
IL-8	Perivascular cells	Premenstrual neutrophil recruitment (Jones, et al., 1997; Milne, et al., 1999)
MCP-1	Perivascular cells	Premenstrual monocyte recruitment (Jones, et al., 1997)
MIP-1α	Glandular epithelium T cells/macrophages	Released from epithelium during menstruation; likely contribution to monocyte/macrophage chemotaxis (Akiyama, et al., 1999)
RANTES	Predominantly stroma	Unclear – T cell/monocyte recruitment? (Hornung, et al., 1997)

B. Cytokines

Inflammatory mediator	Reported source in endometrium	Suggested function in endometrium
Cytokines		
Interferon γ (IFNγ)	T cells in lymphoid aggregates Polymorphonuclear leukocytes	Inhibits epithelial proliferation Immune responsiveness (Tabibzadeh, 1994; Yeaman, et al., 1998)
IL-1	Epithelium Isolated stromal cells Endothelium	Implantation e.g. blastocyst-endometrial communication Menstruation e.g. induction of MMPs, cytokines (Simon, et al., 1993a; Tabibzadeh and Sun, 1992)
IL-6	Glandular & surface epithelium Stroma (late secretory)	Implantation Menstruation (Tabibzadeh, et al., 1995a)
LIF	Glandular epithelium	Endometrial-blastocyst interactions (Charnock-Jones, et al., 1994)
TNFα	Predominantly glandular epithelium (Stroma)	Regulation of DNA synthesis Tissue remodeling Pro-apoptotic Compromises vascular integrity (Hunt, 1993; Tabibzadeh, 1996; Tabibzadeh, et al., 1995b)

C. Growth Factors

Inflammatory mediator	Reported source in endometrium	Suggested function in endometrium
Growth Factors		
Epidermal growth factor (EGF)	Epithelium Stroma	Mitogenesis Differentiation/decidualization (Haining, et al., 1991; Hofmann, et al., 1991)
Insulin-like growth factor	Stroma	Mitogenesis Differentiation/decidualization (Giudice, et al., 1993; Zhou, et al., 1994)
Transforming growth factor α (TGFα)	Surface epithelium Stroma	Interactions with endometrium and blastocyst (Bush, et al., 1998)
TGFβ	All uterine cell types	Angiogenesis during menstruation, inhibition of proliferation, suppression of MMP secretion (Bruner, et al., 1995; Casslen, et al., 1998; Chegini, et al., 1994)

D. Other inflammatory molecules

Inflammatory mediator	Reported source in endometrium	Suggested function in endometrium
Other molecules		
COX-2 (prostaglandin synthesis)	Perivascular cells Glandular & luminal epithelium	Synergistic role of PGE_2 and IL-8 in neutrophil recruitment Vasoactive effects in implantation and menstruation Immunosuppressive effects (Baird, et al., 1996; Jones, et al., 1997; Marions and Danielsson, 1999)
Endothelins	Glandular & luminal epithelium Stroma Some endothelial cells	Vasoactive effects in menstruation (Cameron, et al., 1993; Collett, et al., 1996; Kubota, et al., 1995; Salamonsen, et al., 1992)
Inducible nitric oxide synthase (iNOS)	Glandular epithelium Vascular smooth muscle	Regulation of uterine blood flow during implantation/menstruation Cytotoxic effects (Telfer, et al., 1997; Tschugguel, et al., 1998; Yoshiki, et al., 2000)
MMPs	Epithelium Stroma Lymphoid cells	Tissue remodelling during implantation and menstruation (Lockwood, et al., 1998; Salamonsen, 1998)

1.3 Regulation of inflammatory mediator expression in endometrium

The primary means of control of inflammatory mediator expression in endometrium is via the effects of the steroid hormones. As described earlier (Figure 1) the hormones, oestradiol and progesterone, are produced in a cyclical manner. The hormones, in common with all steroids, act via binding to intracellular receptors belonging to the steroid receptor superfamily. The receptors for both oestrogen (ER) and progesterone (PR) belong to this family. In the absence of hormone the receptors are inactive and are present in complexes containing other proteins such as heat shock protein 90. In the event of hormone binding the receptor dissociates from the complex and can bind to specific hormone response elements in DNA thus altering gene expression. (Carson-Jurica, et al., 1990). The PR and the glucocorticoid receptor (GR) bind to the same consensus sequences (glucocorticoid/progesterone response element; GRE/PRE) and it is thought that, in the uterus, progesterone has local immunosuppressive effects similar to the systemic effects of the glucocorticoids. Also, nonconsensus sequences (negative GRE = nGRE; these differ from typical GRE and have gene repressive function) exist which allow binding of the PR or GR to suppress gene transcription. It should also be noted that, when present in high concentrations, steroid hormones can act via non-genomic mechanisms e.g. modulation of ion channel function (for review see Revelli, et al. (1998)).

The progesterone receptor was first identified in the chick oviduct and mammalian uterus in the 1970s (Leavitt and Blaha, 1972; Milgrom, et al., 1970; Sherman, et al., 1970) and it is now apparent that the human progesterone receptor exists in two forms, PR_A and PR_B (Horwitz and Alexander, 1983; Lessey, et al., 1983). Both forms are transcribed from a single gene under the influence of separate promoters (Kastner, et al., 1990). PR_B is the larger isoform with 164 additional amino acids at the N terminus; other than this the proteins are identical. Both forms of PR are activated by progesterone but they are thought to have differing functions. PR_B is the more transcriptionally active isoform (Wen, et al., 1994) and in settings where both the A and B forms are present PR_A can act as a dominant repressor of PR_B function

(Tung, et al., 1993; Vegeto, et al., 1993). This suggests that in cells where there is an abundance of PR_A the responsiveness to progesterone may be reduced and that the ratio of PR_A: PR_B may determine the cellular response to the hormone. PR_A has also been shown to inhibit the function of the glucocorticoid, mineralocorticoid, androgen and oestrogen receptors (McDonnell and Goldman, 1994; McDonnell, et al., 1994; Wen, et al., 1994).

The oestrogen receptor also exists in two forms: ERα (Green, et al., 1986) and ERβ (Kuiper, et al., 1996; Mosselman, et al., 1996). *In vitro* both proteins are functional and both bind oestradiol with a high affinity (Kuiper and Gustafsson, 1997; Mosselman, et al., 1996).

1.3.1 Oestrogen and progesterone receptor expression in endometrium

Several studies have described the immunohistochemical distribution of the oestrogen and progesterone receptors throughout the menstrual cycle (Critchley, et al., 1993; Lessey, et al., 1988; Snijders, et al., 1996). These studies have shown that ER levels in both endometrial epithelium and stroma increase throughout the proliferative phase peaking at the time of ovulation. Levels then decline in both compartments during the secretory phase. This is consistent with the view that oestrogen positively regulates its receptor while progesterone suppresses ER expression. PR is shown to increase in epithelium and stroma during the proliferative phase (under the influence of oestrogen). During the secretory phase PR expression decreases in the epithelium but persists in stromal cells, particularly those in the perivascular region (Wang, et al., 1998). In pregnancy PR remains in the perivascular and stromal cells while ER is absent. Both ER and PR are absent from epithelium (Perrot-Applanat, et al., 1994).

Recently, the distribution and cycle dependence of the two PR isoforms has been described by further immunohistochemical studies (Mote, et al., 1999; Wang, et al., 1998). These studies have shown that, while PR_A and PR_B are coexpressed in glandular epithelium and stroma in the proliferative phase, mainly PR_A is present in

the stroma in the secretory phase and early pregnancy. This suggests that PR_A is the isoform responsible for decidualization and other stromal events influenced by progesterone. Also, the localization of both PR isoforms to the predecidual cells around the spiral arterioles suggests that progesterone withdrawal effects may be profound in these cells (Wang, et al., 1998). This would be in keeping with the upregulation of IL-8, MCP-1 and COX-2 reported in these cells in the premenstrual phase. Both PR isoforms are reported to decrease in epithelium in the secretory phase. However, PR_B is reported to be present in the mid secretory phase and it is suggested that this isoform may be responsible for the progestogenic effects which cause glandular secretion (Mote, et al., 1999).

The localization of ERα and ERβ proteins in the endometrium has also recently been reported. Both ERα and ERβ have been detected in glandular epithelium, stroma and perivascular cells. Decreased expression of ERβ in glands in the functionalis region has been documented in the late secretory phase. ERβ is also present in the endometrial endothelium and it is suggested that this receptor may mediate steroid effects on the endometrial vasculature (Critchley, et al., 2000). A further study detailed the localization of mRNA for the two isoforms by in-situ hybridization (Matsuzaki, et al., 1999). ERα mRNA was expressed by glandular epithelial and stromal cells. This expression decreased in both cell types in the functionalis layer during the secretory phase of the cycle although stromal cells near secretory glands continued to express ERα mRNA. This was consistent with immunohistochemical results. ERβ mRNA was expressed predominantly by the glandular epithelium with only faint stromal staining. Again, decreased expression was apparent in the secretory phase of the cycle. This study suggested that oestrogenic effects on the endometrium are likely to occur mainly via the ERαisoform although glandular effects may also involve ERβ.

Although the steroid hormones exert a fundamental influence on the regulation of inflammatory mediator expression in endometrium the precise molecular mechanisms involved remain unclear. The promoter regions for several cytokines do not express GRE/PRE suggesting that progesterone cannot act directly to suppress expression

and, equally, progesterone withdrawal cannot directly influence upregulation (McKay and Cidlowski, 1999). Also, the focal expression of many inflammatory mediators indicates that a more subtle mechanism is involved in their expression. Cross-talk between the steroid hormone signal transduction pathways and other pathways is probable. The nuclear factor kappa B (NFκB) signal transduction pathway is likely to be involved in such interactions.

1.3.2 Nuclear factor kappa B (NFκB)

NFκB is a transcription factor involved in the inflammatory and immune response (Baldwin, 1996; Siebenlist, et al., 1994). It was first identified in B cells as a nuclear factor which bound to the immunoglobulin κ enhancer (Sen and Baltimore, 1986) and subsequently, it has been established that NFκB binding sites are present in the promoter regions of genes such as IL-1, IL-6, class I MHC and COX-2. Table 2 details molecules relevant to endometrial function which are regulated by NFκB. NFκB belongs to the Rel family of transcription factors which all contain an approximately 300 amino acid Rel homology domain. This is involved in DNA binding, dimerization with other Rel proteins and binding with inhibitor of NFκB (IκB) protein family members. There are currently several members of the NFκB/Rel family of transcription factors: c-Rel, NFκB1 (p50/p105), NFκB2 (p52/p100), Rel A (p65), Rel B and the *Drosophila* protein, dorsal. These proteins form both homo- and heterodimers with the classic NFκB heterodimer consisting of p50 and p65. NFκB binds to the consensus sequence 5`-GGGPuNNPyPyCC-3` thus activating gene transcription. It should be noted that NFκB rarely acts alone when regulating gene transcription. Instead, there is likely to be complex interactions between NFκB and other transcription factors (for review see McKay and Cidlowski (1999)). As indicated above, there is cross-talk between steroid hormones and NFκB (described in section 1.3.6) and activator protein 1 (AP-1), another transcription factor involved in the immune response, often acts in synergy with NFκB.

Table 2: Examples of NFκB regulated inflammatory molecules. Those molecules which are also shown in Table 1 are highlighted in red. It can be seen that many of the genes induced by NFκB are expressed by human endometrium.

Gene induced by NFκB	Reference
COX-2	(Adcock, et al., 1997)
eotaxin	(Matsukura, et al., 1999)
Granulocyte/macrophage colony stimulating factor (GM-CSF)	(Adcock, et al., 1997)
IFNγ	(Sica, et al., 1997)
IL-1β	(Hiscott, et al., 1993)
IL-6	(Gruss, et al., 1992)
IL-8	(Mukaida, et al., 1990)
iNOS	(Chao, et al., 1997)
Interferon regulatory factor 1 (IRF-1)	(Liu, et al., 1999)
IκBα	(Sun, et al., 1993)
MCP-1	(Ueda, et al., 1994)
MIP-1α	(Grove and Plumb, 1993)
MMP-1/MMP-9	(Bond, et al., 1998; Vincenti, et al., 1998; Yokoo and Kitamura, 1996)
RANTES	(Manni, et al., 1996)
TNFα	(Shakhov, et al., 1990)

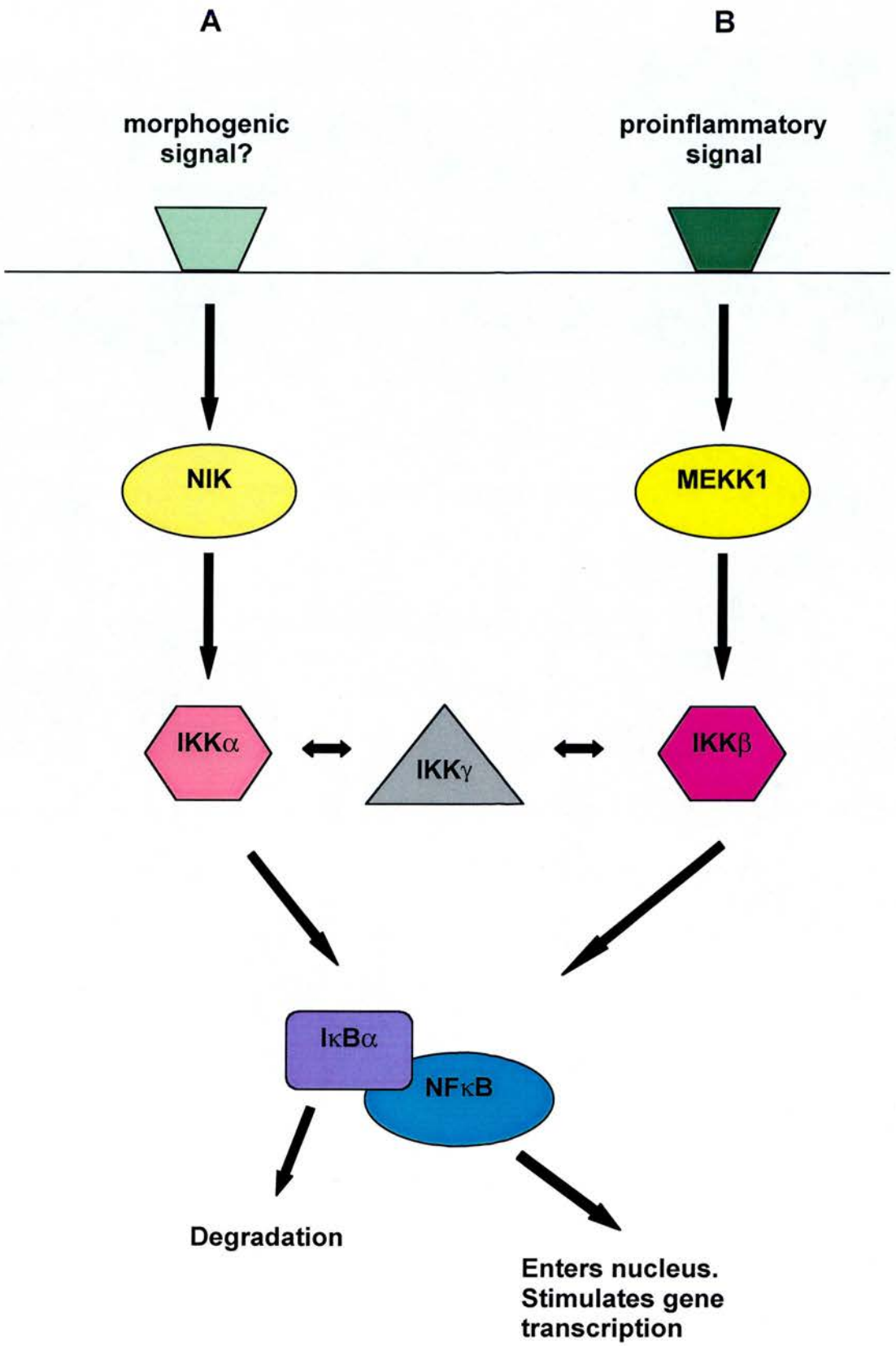
NFκB is held inactive in the cytoplasm by endogenous inhibitors belonging to the IκB family (Baeuerle and Baltimore, 1988; Baeuerle, et al., 1988). These proteins are also responsible for inhibiting the DNA binding of NFκB. Initially, two IκB proteins were identified, IκBα(MAD-3) and IκBβ (Zabel and Baeuerle, 1990). Subsequently, other family members have been found including IκBε, IκBγ, Bcl-3 and the *Drosophila* protein cactus. Additionally, the Rel proteins p105 and p100 can act as inhibitors of NFκB. The IκB proteins contain ankyrin repeats (33 amino acid sequences, originally found in the erythrocyte protein ankyrin) which are responsible for their interaction with the Rel domain of NFκB and it is now apparent that IκB retains NFκB in the cytoplasm by masking its nuclear localization sequence. Recent crystallographic studies have confirmed that loops between the ankyrin repeats contain the residues crucial for NFκB binding and that Rel A undergoes a conformational change, masking DNA binding residues, when bound to IκBα (Huxford, et al., 1998; Jacobs and Harrison, 1998). Differential binding of the IκB proteins to the various NFκB dimers is observed with IκBα binding preferentially to p50-p65 and p50-c-Rel heterodimers (Thompson, et al., 1995). Differential targeting of IκBα and IκBβ has also been reported with IκBβ targeted only by a subset of NFκB stimulators. While the presence of multiple NFκB and IκB proteins is acknowledged, the following discussion of the signalling pathway will focus mainly on the classic NFκB (p50/p65) heterodimer and IκBα.

1.3.3 The NFκB signal transduction pathway – The IKK complex

The NFκB signal transduction pathway, as described in the following sections (1.3.3 and 1.3.4), is illustrated in Figure 4. Upon stimulation of the cell, IκBα becomes phosphorylated (Beg and Baldwin, 1993) on Ser 32 and 36 (Brockman, et al., 1995; Brown, et al., 1995) and then ubiquinated on Lys 21 and 22 (Scherer, et al., 1995) with subsequent dissociation from NFκB. This allows the release of free NFκB which travels to the nucleus where it can regulate gene expression while free IκBα is rapidly degraded via a proteasome dependent pathway. Crucially, one gene upregulated by NFκB is IκBα allowing replenishment of the IκB stock and self-

limitation of NFκB activation (Sun, et al., 1993). The pathway leading to IκBα phosphorylation has recently been identified (for reviews see Hatada, et al., 2000; Israel, 2000; Mercurio and Manning, 1999; Stancovski and Baltimore, 1997; Zandi and Karin, 1999).

Figure 4: The NFκB signal transduction pathway. An extracellular signal activates the transcription factor, NFκB, via a series of protein kinase. The result is phosphorylation of the inhibitor, IκBα, followed by translocation of NFκB to the nucleus. IκB kinase α (IKKα) is believed to mediate morphogenic signalling to NFκB and NFκB inducing kinase (NIK) is thought to preferentially phosphorylate this kinase (Pathway A). In contrast, IκB kinase β (IKKβ) is believed to be primarily involved in proinflammatory signalling to NFκB and is preferentially phosphorylated by mitogen activated protein kinase/ERK kinase kinase 1 (MEKK1) (Pathway B).


A
B
morphogenic signal?
proinflammatory signal
NIK
MEKK1
IKKα
IKKγ
IKKβ
IκBα
NFκB
Degradation
Enters nucleus. Stimulates gene transcription

1.3.3a Identification of IKKα and IKKβ

In 1996 it was reported that an IκB kinase (IKK) complex had been purified from the cytoplasm of unstimulated HeLa cells (Chen, et al., 1996). The complex was found to phosphorylate IκBα at the relevant Ser residues *in vitro*. Following this, two kinases were detected in a similar IKK complex (this complex was purified from cells which had been stimulated with TNFα) with the subsequent identification of IKKα (also known as IKK-1; conserved helix-loop-helix ubiquitous kinase) and IKKβ (IKK-2) (DiDonato, et al., 1997; Mercurio, et al., 1997; Regnier, et al., 1997; Woronicz, et al., 1997; Zandi, et al., 1997). The proteins were found to be approximately 50% homologous, both had N terminal kinase domains and contained a leucine zipper domain and a helix-loop-helix motif. These domains have been found to be involved in interactions between the kinases and other molecules in the IKK complex. IKKα and IKKβ are believed to associate both as heterodimers and homodimers *in vitro* (Mercurio, et al., 1997; Woronicz, et al., 1997; Zandi, et al., 1998; Zandi, et al., 1997). *In vivo* Woronicz *et al* reported the presence of heterodimers (Woronicz, et al., 1997) while Mercurio *et al* detected both heterodimers and IKKβ homodimers (Mercurio, et al., 1999). Mutations in the leucine zipper domain of the kinases has been shown to prevent dimerization (Woronicz, et al., 1997; Zandi, et al., 1997) and the helix-loop-helix motif is thought to mediate interactions with other molecules in the IKK complex (Zandi, et al., 1997). Mutational studies have also shown that both the kinase domain and the leucine zipper motif of IKKβ are needed for interactions with IκBα (Woronicz, et al., 1997).

Both IKKα and IKKβ activity is reported to cause phosphorylation of IκBα on Ser 32 and Ser 36 (mutant forms of IκBα are not phosphorylated with the same efficiency) (DiDonato, et al., 1997; Mercurio, et al., 1997; Woronicz, et al., 1997). Subsequently, experiments transfecting IKKα and IKKβ into insect cells have shown direct phosphorylation of IκB by both kinases (Zandi, et al., 1998). This study also demonstrated that the kinases preferentially phosphorylate IκB proteins that are bound to NFκB rather than free IκB. This is consistent with the physiological

substrate for the IKK complex being NFκB complexed IκB. Activators of NFκB (e.g. IL-1, TNFα) induced IKK kinase activity with kinetics similar to those seen for phosphorylation of IκBα (DiDonato, et al., 1997; Mercurio, et al., 1997; Zandi, et al., 1997) while perturbation of kinase expression or activity provided further evidence that these kinases are involved in NFκB activation. Overexpression of IKKα (DiDonato, et al., 1997) and IKKβ (Woronicz, et al., 1997) caused increased activation of NFκB by IL-1 and TNFα. In contrast, kinase inactive mutants of IKKα or IKKβ and antisense RNA against IKKα inhibited NFκB activation by IL-1 and TNFα (DiDonato, et al., 1997; Mercurio, et al., 1997; Woronicz, et al., 1997). Several studies have reported incomplete inhibition of NFκB activation by kinase inactive mutants (Mercurio, et al., 1997; Zandi, et al., 1998; Zandi, et al., 1997).

1.3.3b Differential functions of IKKα and IKKβ

Although both IKK proteins have been shown to be capable of NFκB activation it is becoming apparent that the proteins have differing activities. IKKβ is reported to be the more active kinase (Mercurio, et al., 1997; Woronicz, et al., 1997) and it is also able to phosphorylate IκBβ equally well on both Ser 19 and 23 (Woronicz, et al., 1997). IKKα preferentially phosphorylates Ser 23 (Regnier, et al., 1997). Recent reports have also suggested that the proteins have different functions (for review see May and Ghosh (1999)). Two groups have generated IKKα deficient mice with both reporting abnormal fetal development (Hu, et al., 1999; Takeda, et al., 1999). The mice showed abnormal limb development, shortened heads and snouts with no external ears and shiny, rather than the normal wrinkled, skin. The skeletal development of the limbs was found to be normal but they remained tightly folded. Differentiation of keratinocytes was prevented and there was hyperplasia of the epidermis. Hu *et al* also reported abnormal development of the sternum and vertebrae. Fibroblasts and thymocytes derived from the mutant embryos had a normal NFκB activation response to stimulation with IL-1 and TNFαand it was assumed that this is mediated via IKKβ. These studies suggest that IKKα is not, as originally believed, critical to NFκB activation by proinflammatory signals but

instead, is involved in early development and the transmission of morphogenic signals to NFκB (see Pathway A, Figure 4).

The role of IKKβ has also been investigated and it has been found that IKKβ deficient mice die as embryos due to apoptosis in the liver. These mice can be rescued by inactivation of the gene for TNFαreceptor 1 suggesting that the cells apoptose as a result of unchallenged activation of pro-apoptotic pathways by TNFα. Normally, NFκB would also be activated and this would have anti-apoptotic effects. Fibroblasts derived from the IKKβ deficient mice showed reduced NFκB activation upon stimulation by IL-1 and TNFα (Li, et al., 1999). Similar results have been reported by Tanaka *et al* (1999). The role of IKKβ has also been investigated by Delhase *et al* (1999). In this study the activation of IKK activity by upstream kinases was investigated using mutational studies. It was found that elimination of two phosphorylation sites within the activation loop (T loop) of IKKβ, but not IKKα, prevented IKK activation by proinflammatory cytokines or the upstream kinases, NFκB inducing kinase (NIK) and mitogen activated protein kinase/ERK kinase kinase-1 (MEKK1; ERK, extracellular regulated kinase). It is thought that when IKKβ becomes activated (due to stimulation by proinflammatory molecules) it may activate IKKα although this is not essential for NFκB activation. Also, IKKβ is involved in negative regulation of IKK activity via autophosphorylation of C terminal residues (Delhase, et al., 1999). These results suggest that IKKβ is the main kinase involved in proinflammatory signalling to NFκB (see Pathway B, Figure 4) and this is consistent with reports that the anti-inflammatory agents aspirin and salicylate inhibit only IKKβ (Yin, et al., 1998).

1.3.3c Additional components of the IKK complex

Further components of the IKK complex have also been described. IKKγ (or IKK associated protein-1) has been identified by two groups (Mercurio, et al., 1999; Rothwarf, et al., 1998) and has been found to be the human homologue of the mouse NFκB essential modulator (NEMO) discovered by Yamaoka (Yamaoka, et al., 1998).

IKKγ has been detected in two forms IKKγ 1 and 2 (Rothwarf, et al., 1998) and is thought to have two coiled-coil motifs and a leucine zipper. IKKγ can interact with both IKKα and IKKβ in cotransfection studies but can only stably interact with IKKβ. IKKγ antisense experiments have shown inhibition of IKK activity upon stimulation with IL-1 or the upstream kinases, NIK and MEKK1. Additionally, inhibition of p65 nuclear translocation upon TNFαstimulation was reported (Rothwarf, et al., 1998). Mutant versions of IKKγ or molecules lacking the IKKβ binding site also inhibit NFκB activation (Mercurio, et al., 1999). IKKγ is identical to a previously identified protein, FIP3, which can interact with the TNFα receptor complex and immunocytochemical studies have shown that the C-terminal portion of IKKγ translocates to the cell membrane upon stimulation with TNFα (Mercurio, et al., 1999). This led the authors to suggest that IKKγ may act as a scaffolding protein allowing interaction of IKKβ containing IKK complexes with upstream components of the NFκB activation pathway (e.g. the TNFα receptor complex). Another likely scaffolding component of the IKK complex, IKK-complex-associated protein (IKAP) has also been identified (Cohen, et al., 1998). IKAP was found to interact with NIK, IKKα and IKKβ with greatest affinity for NIK. Endogenous NIK and IKKβ were found to exist in a complex with IKAP and it was suggested that IKAP promotes an interaction between the IKK complex and the upstream activating kinase, NIK.

1.3.4 The NFκB signal transduction pathway – Upstream of the IKK complex

Several kinases have been implicated in the phosphorylation of the IKK complex. The mitogen activated protein kinase kinase kinase enzymes, NIK and MEKK1, have both been identified as kinases involved in NFκB activation (see Figure 4). NIK was identified due to its ability to bind to TNF receptor associated factor 2 (TRAF2) which is a protein crucial for NFκB activation by TNFα (Malinin, et al., 1997). It has also been reported to interact with TRAF6 (Song, et al., 1997). Expression of a kinase inactive NIK mutant blocked activation of NFκB due to TNFαand IL-1 stimulation (Malinin, et al., 1997). MEKK1 was identified as an NFκB activating kinase by similar experiments (Lee, et al., 1997; Lee, et al., 1998; Nemoto, et al.,

1998) . It has been reported that NIK and MEKK1 preferentially phosphorylate IKKα and IKKβ, respectively (Ling, et al., 1998; Nakano, et al., 1998) (see Pathways A & B, Figure 4). MEKK2 and 3 have also been shown to be capable of stimulating IKK activity and activation of NFκB (Zhao and Lee, 1999).

The TNF and IL-1 receptors connect to the NFκB activation pathway through the actions of several proteins. Upon binding of IL-1 to the IL-1 RtI a complex is formed between IL-1R accessory protein, myeloid differentiation factor 88 and a serine/threonine kinase, IL-1 receptor associated kinase (IRAK). IRAK dissociates from the receptor and has been found to interact with TRAF6. TRAF6 then interacts with the protein kinases, transforming growth factor β activated kinase 1 (TAK1) and TAK1 binding protein 1, resulting in NIK activation (Cao, et al., 1996; Ninomiya-Tsuji, et al., 1999; Wesche, et al., 1997). In the case of TNF receptor 1 (TNFR1), the binding of TNFα results in the oligomerization of death domains present in the cytoplasmic regions of the receptor. This allows recruitment of several proteins including TNFR-associated death domain protein, TRAF2 and receptor-interacting protein (Hsu, et al., 1996; Hsu, et al., 1995; Rothe, et al., 1994). As detailed above TRAF2 is thought to associate with NIK.

A TRAF-binding protein, TRAF family member associated NFκB activator (TANK; or TRAF interacting protein), reported to inhibit the activation of NFκB by TNFα (via competition with TRAFs for binding sites on the cytoplasmic tail of the TNFR2), has also been found to be involved in an NFκB activation pathway. An IKK-like kinase, TANK binding kinase 1 (TBK1), interacts with TANK in a TBK1-TANK-TRAF2 complex. This interaction with TRAF2 allows signalling to NFκB via the NIK-IKK pathway (Pomerantz and Baltimore, 1999). Interestingly, TBK1 is 48% homologous to an inducible IKK which has been identified in mouse macrophages (Shimada, et al., 1999).

It should be noted that the elucidation of the NFκB signalling pathway has focused mainly on stimulation by IL-1 and TNFα. However, activation by most other stimuli is believed to occur via the IKK complex.

1.3.5 Activators of NFκB

As described above NFκB is activated by IL-1 and TNFα. Further stimuli of relevance to the female reproductive system are described in Table 3.

Table 3: Examples of molecules thought to activate the NFκB signal transduction pathway. Several of these molecules (shown in red) are expressed by human endometrium and are also detailed in Table 1. Others (e.g. HIV-1) are sexually transmitted diseases suggesting that NFκB activation may be involved in the mechanism of infection in the reproductive tract.

Stimulus	Reference
Endothelin-1	(Browatski, et al., 2000; Gallois, et al., 1998)
GM-CSF	(Oster, et al., 1992)
Herpes simplex virus 1	(Patel, et al., 1998)
HIV-1	(Bachelerie, et al., 1991)
IFNγ	(Narumi, et al., 1992)
IL-1α/β	(Osborn, et al., 1989)
Integrins	e.g. (Bhullar, et al., 1998) $\alpha_v\beta_3$ (Udagwa, et al., 1996) $\alpha_4\beta_1$
LIF	(Gruss, et al., 1992)
Lipopolysaccharide (LPS)	(Richardson, et al., 1991)
Macrophage-colony stimulating factor	(Oster, et al., 1992)
PGE_2	(Muroi and Suzuki, 1993)
TNFα	(Osborn, et al., 1989)

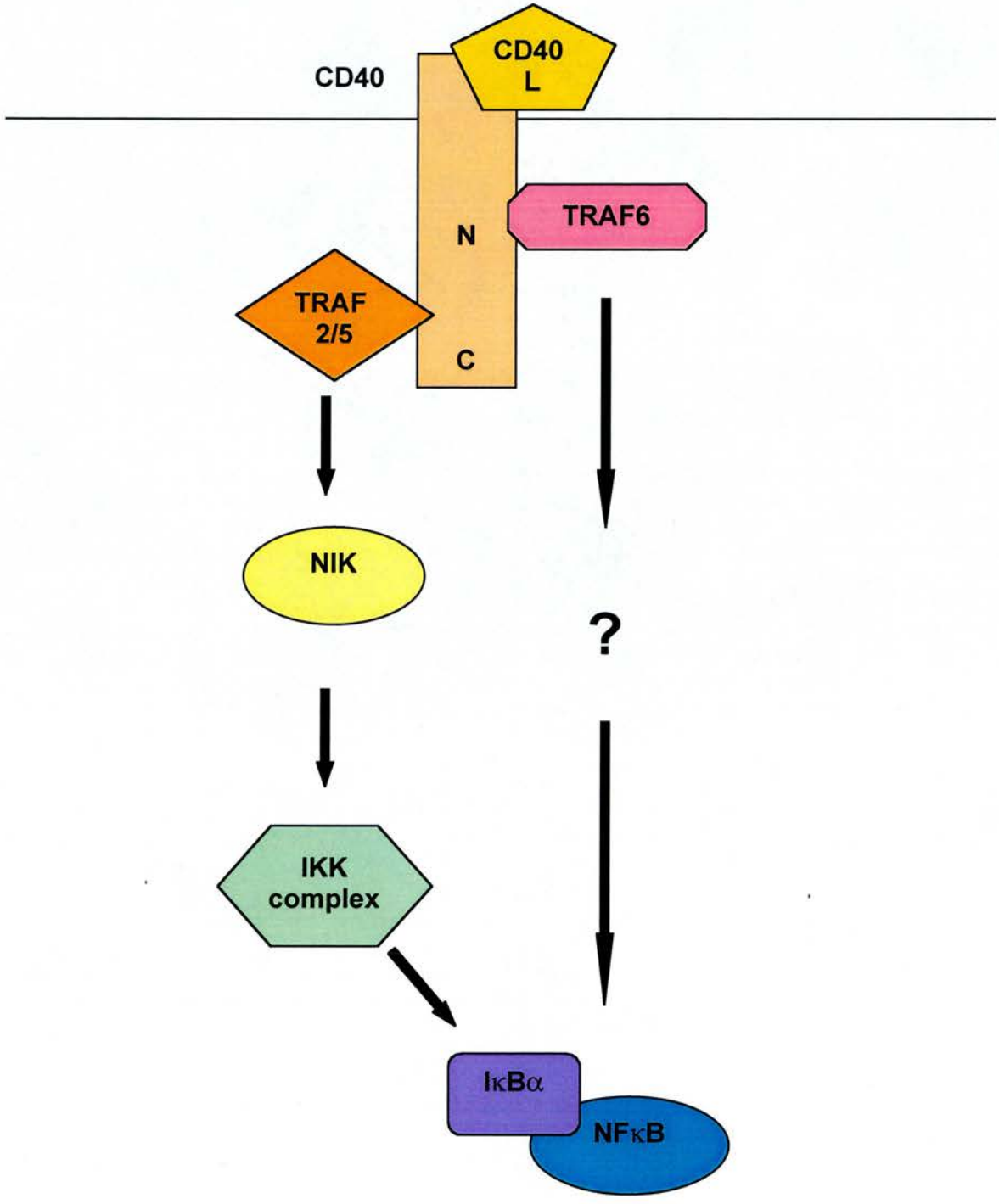
1.3.5a The CD40-CD40 ligand system

CD40, a member of the TNF receptor superfamily, was originally detected on the surface of B cells (Stamenkovic, et al., 1989) and has a role in the activation, proliferation and differentiation of these cells when activated by its ligand, CD40 ligand (CD40L/wCD154), found on the surface of T cells (Graf, et al., 1992). Subsequently, it has been discovered that both CD40 and CD40L are expressed on the surface of multiple cells types. CD40 has been reported to be present on monocytes (Alderson, et al., 1993), fibroblasts (Fries, et al., 1995) and dendritic (Freudenthal and Steinman, 1990), endothelial (Hollenbaugh, et al., 1995) and some epithelial cells (Galy and Spits, 1992). In addition to T cells, CD40L is expressed on eosinophils (Gauchat, et al., 1995), basophils, mast cells (Gauchat, et al., 1993), activated platelets (Henn, et al., 1998), NK cells (Carbone, et al., 1997) and macrophages (Mach, et al., 1997).

In non-haematopoietic cells the CD40-CD40L system has been found to be involved in proinflammatory signalling and activation of the pathway has been reported to stimulate cytokine release by several cell types. Upregulation of cytokines including IL-1, IL-6, IL-8, IL-10, TNFα and MIP-1α upon crosslinking of CD40 on monocytes has been shown (Caux, et al., 1994; Kiener, et al., 1995). Similarly, activation of CD40 has been found to increase production of IL-6 and IL-8 by fibroblasts from several sites (e.g. orbital, lung) (Sempowski, et al., 1997a; Sempowski, et al., 1998). COX-2 and PGE_2 are also upregulated by CD40 stimulation of lung fibroblasts (Zhang, et al., 1998). In endothelial cells CD40 is involved in the increased expression of adhesion molecules including vascular and intercellular adhesion molecules (VCAM-1 and ICAM-1) (Hollenbaugh, et al., 1995; Karmann, et al., 1995). These results suggest a role for the CD40 system in control of inflammatory mediator expression and leukocyte migration during inflammatory events. Prior to the results presented in this thesis there were no reports detailing CD40 expression and action in the endometrium.

CD40 is an activator of NFκB signal transduction (Berberich, et al., 1994). It is also involved in modulation of nonreceptor tyrosine kinase activity, phosphorylation of phospholipase Cγ2, activation of phosphatidylinositol-3-kinase and stimulation of the Janus kinase – signal transducer and activator of transcription (JAK-STAT) pathway (Faris, et al., 1994; Hanissian and Geha, 1997; Ren, et al., 1994; Schoenberger, et al., 1998). The molecular pathways leading from CD40 to NFκB activation are believed to be similar to those described for TNFα. CD40 has been reported to associate with TRAF2 (Rothe, et al., 1994), TRAF3 (Hu, et al., 1994), TRAF5 (Ishida, et al., 1996b) and TRAF6 (Ishida, et al., 1996a). Recently, it has been shown that the N and C terminal portions of the CD40 cytoplasmic tail interact differentially with the TRAFs. The N terminus interacts with TRAF6 while the other 3 TRAF molecules associate with the C terminus. Mutation of threonine 254 in the C terminus prevents binding of TRAFs to this part of CD40 (Hu, et al., 1994). Similarly, it has now been established that mutation of a glutamate residue in the N terminus prevents binding of TRAF6. The coupling of CD40 to NIK via these adapter molecules has also been investigated. The use of dominant negative NIK mutants (mutant protein that prevents the wild type copies of the protein from performing their normal function) has shown that TRAF2 and TRAF5 activate NFκB via stimulation of NIK while TRAF6 acts mainly via a different, unidentified pathway (Tsukamoto, et al., 1999). This suggests that signalling from CD40 can activate NFκB by at least two different mechanisms (Figure 5).

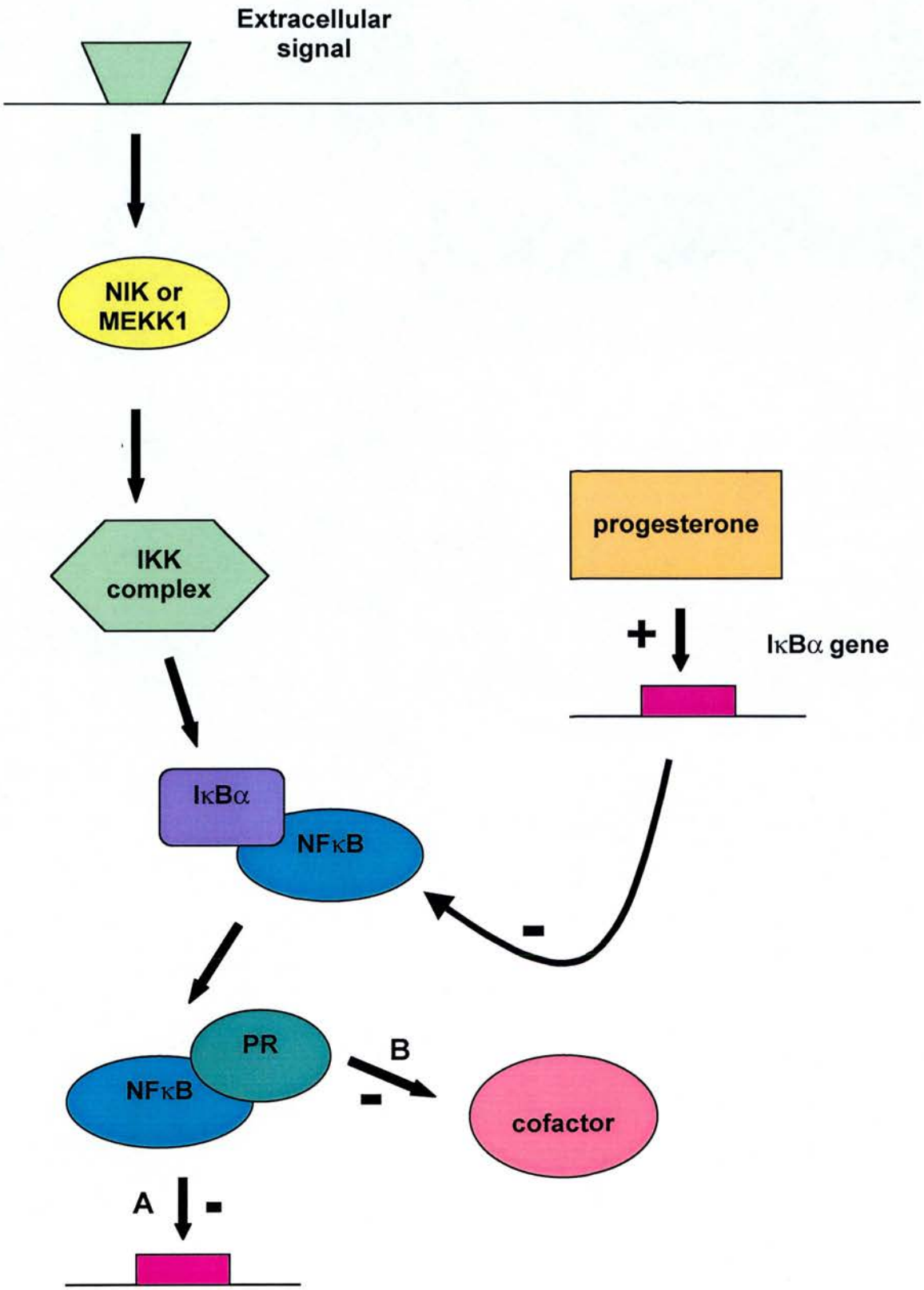
Figure 5: CD40-CD40L activation of NFκB. CD40 activates NFκB by at least two distinct pathways. (1). TRAF 2 and 5 interact with the C terminal region of the CD40 cytoplasmic tail (C) and are involved in signal transduction to NFκB via stimulation of NIK. (2). TRAF 6 interacts with the N terminus of the cytoplasmic tail (N) and is involved in NFκB activation via an unidentified pathway.


CD40
CD40
L
N
C
TRAF6
TRAF
2/5
NIK
?
IKK
complex
IκBα
NFκB

1.3.6 Inhibitors of NFκB

Glucocorticoids, the anti-inflammatory steroid hormones, inhibit the activity of NFκB and it is thought that it is partly via this action that they suppress the production of inflammatory mediators such as IL-1 and IL-8. The suppression of NFκB occurs via several mechanisms (McKay and Cidlowski, 1999). First, glucocorticoids have been shown to increase the mRNA and protein levels of IκBα (Auphan, et al., 1995; Scheinman, et al., 1995a). This will cause the increased retention of NFκB in the cytoplasm. Second, the GR has been shown to directly interact with the p65 subunit of NFκB resulting in mutual inhibition (Caldenhoven, et al., 1995; McKay and Cidlowski, 1998; Ray and Prefontaine, 1994; Scheinman, et al., 1995b). This interaction is thought to involve the Rel homology domain of p65 and the DNA binding domain of the GR. The observation that overexpression of p65 does not prevent GR mediated repression of GR expression (McKay and Cidlowski, 1998) suggests that the mutual inhibitory effects may occur by indirect mechanisms such as competition for a common cofactor. As mentioned earlier, progesterone has local immunosuppressive effects similar to those of glucocorticoids. Progesterone has also been reported to interact with the NFκB pathway. Increased IκBα gene expression in the presence of progesterone has been reported in the T47D cell line and in mouse macrophages (Miller and Hunt, 1998, Wissink, 1998) and direct interactions between p65 and the PR have also been reported (Kalkhoven, et al., 1996; McKay and Cidlowski, 1998). However, there were discrepancies between these studies with only Kalkhoven *et al* reporting mutual inhibition; McKay *et al* found only inhibition of PR by p65. The reduced level of inflammatory mediator expression in the secretory phase of the menstrual cycle and pregnancy may be mediated in part by progesterone suppression of the NFκB pathway. Additionally, progesterone withdrawal premenstrually may release inhibition of NFκB allowing upregulation of inflammatory molecules. Progesterone inhibition of the NFκB pathway is demonstrated in Figure 6.

Figure 6: Progesterone interactions with the NFκB pathway. Progesterone increases the expression of the endogenous inhibitor, IκBα. The progesterone receptor can interact with NFκB preventing binding to DNA either **(A)** directly or **(B)** via competition for a common cofactor.


Extracellular signal
NIK or MEKK1
IKK complex
progesterone
+
IκBα gene
IκBα
NFκB
-
PR
NFκB
B
-
cofactor
A
-

1.3.6a Secretory leukocyte protease inhibitor (SLPI)

SLPI (also known as antileukoprotease) is an 11.7kDa cysteine rich protein expressed by neutrophils (Bohm, et al., 1992), macrophages (Jin, et al., 1997) and epithelial cells (Abe, et al., 1991). It has been found associated with mucosal surfaces such as lung and cervix (Franken, et al., 1989) and has been characterized as a crucial neutrophil elastase inhibitor in lung. SLPI has also been reported to have inhibitory effects on another neutrophil protease, cathepsin G, and the pancreatic enzymes, trypsin and chymotrypsin (Thompson and Ohlsson, 1986). Several disease states are characterized by proteolytic tissue damage (e.g. cystic fibrosis, asthma) and SLPI has been used as a potential treatment for such conditions (Birrer, 1995). In addition to its role as a serine protease inhibitor, SLPI has several other functions that suggest that it may have a more generalized protective effect at mucosal surfaces. SLPI has been reported to have antibacterial, antiviral and antifungal effects (Tomee, et al., 1998). SLPI has antibacterial actions against *Escherichia coli* and *Staphylococcus aureus in vitro* suggesting that it has bactericidal effects on both Gram negative and positive micro-organisms (Hiemstra, et al., 1996). SLPI also has antibacterial effects against micro-organisms found on skin (Wiedow, et al., 1998). The mechanism by which SLPI has these actions is unclear but it has been suggested to bind to bacterial mRNA and DNA (Miller, et al., 1989). Also, it is possible that the cationic nature of SLPI may be involved as higher salt concentrations inhibit its bactericidal activity (Hiemstra, et al., 1996). Interestingly, SLPI has two domains that show high homology to each other. The C terminal domain is responsible for the protease inhibitory effects (Eisenberg, et al., 1990; Kramps, et al., 1990) while the N terminus has been shown to be involved in the bactericidal effects of the molecule (Hiemstra, et al., 1996). The N terminal domain is also thought to be responsible for anti-fungal effects (Tomee, et al., 1997). The fungi, *Aspergillus fumigatus* and *Candida albicans*, are killed by SLPI when they are metabolically active. Antiviral effects have also been demonstrated with SLPI, present in saliva, reported to reduce the infection of monocytes with HIV-1 (McNeely, et al., 1995).

SLPI also acts to inhibit various proinflammatory events. For example, in mouse macrophages transfection of SLPI results in suppression of LPS effects (NFκB activation, TNFα and nitric oxide production) (Jin, et al., 1997). SLPI also suppresses MMP production in human monocytes (Zhang, et al., 1997). This occurred via a downregulation of COX-2 expression and hence, PGE_2 production. The mechanism by which SLPI decreases COX-2 expression is not clear but inhibitory effects on NFκB activation have been reported (Jin, et al., 1997; Lentsch, et al., 1999). It has been suggested that SLPI inhibits NFκB via an elevation of IκBβ protein (Lentsch, et al., 1999). Additionally, a SLPI receptor has been identified on human monocytes (McNeely, et al., 1997).

SLPI expression is reported to be upregulated by LPS, IL-6 and IL-10 in mouse macrophages (Jin, et al., 1998) and by phorbol, 1-myristate, 13-acetate (PMA), IL-1β and TNFα in bronchial epithelial cells (Maruyama, et al., 1994; Sallenave, et al., 1994). IFNγ has also been suggested to regulate SLPI expression via IRF-1 although high levels of IRF-1 causes suppression of SLPI levels (Nguyen, et al., 1999). Several of these mediators can activate NFκB and it has been suggested that SLPI may be an NFκB responsive gene (Nguyen, et al., 1999). The increased expression of SLPI by proinflammatory mediators may ensure the presence of protective molecules at inflammatory sites. SLPI secretion is also increased by glucocorticoids in airway epithelial cells (Abbinante-Nissen, et al., 1995). Consistent with this progesterone has been reported to increase SLPI secretion by cervical explants (Denison, et al., 1999a).

SLPI is, therefore, an anti-inflammatory molecule that is able to inhibit the NFκB pathway. Additionally, SLPI is likely to be upregulated by activators of NFκB and also, by anti-inflammatory steroids such as progesterone. Although SLPI has been detected in human seminal plasma (Franken, et al., 1989) and cervical mucosa (Casslen, et al., 1981) previous reports have been unable to detect the molecule in human endometrium (Casslen, et al., 1981; Franken, et al., 1989).

1.4 Hypothesis and Aims

Previous studies on the mechanisms involved in the inflammatory-like events associated with menstruation and implantation have focused on the role of molecules such as chemokines, matrix metalloproteinases and vasoactive agents. While the expression of such molecules is thought to be controlled primarily by the steroid hormones, oestradiol and progesterone, the molecular mechanisms involved have yet to be defined.

The NFκB pathway, due to its involvement in proinflammatory signalling and suppression by progesterone, is likely to be involved in the inflammatory events in endometrium. Although progesterone has been found to suppress the NFκB pathway at several levels there is no data detailing its effects on the expression of intermediate molecules involved in the signalling pathway. Similarly, there is little information regarding the role of NFκB in the endometrium. However, comparison of Tables 1, 2 and 3 shows that many of the inflammatory mediators involved in endometrial function are either regulated by NFκB or interact with the NFκB pathway. High progesterone levels, such as those found in the secretory phase and early pregnancy, would be expected to repress signalling via the NFκB pathway (Figure 7a). This would contribute to the immunosuppressive effects necessary for successful implantation and pregnancy. In contrast, progesterone withdrawal premenstrually would result in relaxation of inhibitory mechanisms and allow NFκB activation (Figure 7b). This would allow upregulation of inflammatory mediator expression.

Although steroid hormones would exercise control over potentially NFκB mediated events in endometrium the production of local mediators would provide a more subtle mechanism of control (partly via feedback loops). For example, the presence of NFκB activators (e.g. CD40) or inhibitors (e.g. SLPI) in endometrium would provide another level of regulation of the pathway. Many mediators, which have been identified as regulators of NFκB, have previously been identified in endometrium (e.g. IL-1). However, as stated above, neither CD40 nor SLPI had been successfully detected in human endometrium prior to the work described in this thesis. The role

of CD40 in upregulation of inflammatory mediators in various cell types suggests that it could play a similar role in menstruation. The expression of CD40L on bone marrow derived cells would provide a mechanism by which leukocytes infiltrating endometrium premenstrually could activate CD40 and upregulate inflammatory molecules. SLPI, as detailed above, is an anti-inflammatory molecule and, if expressed by endometrium, would be expected to contribute to the immunosuppressive events surrounding pregnancy. Figure 7 details the hypothesized involvement of the NFκB pathway, CD40 and SLPI in endometrial physiology.

The aims of this research project are thus:

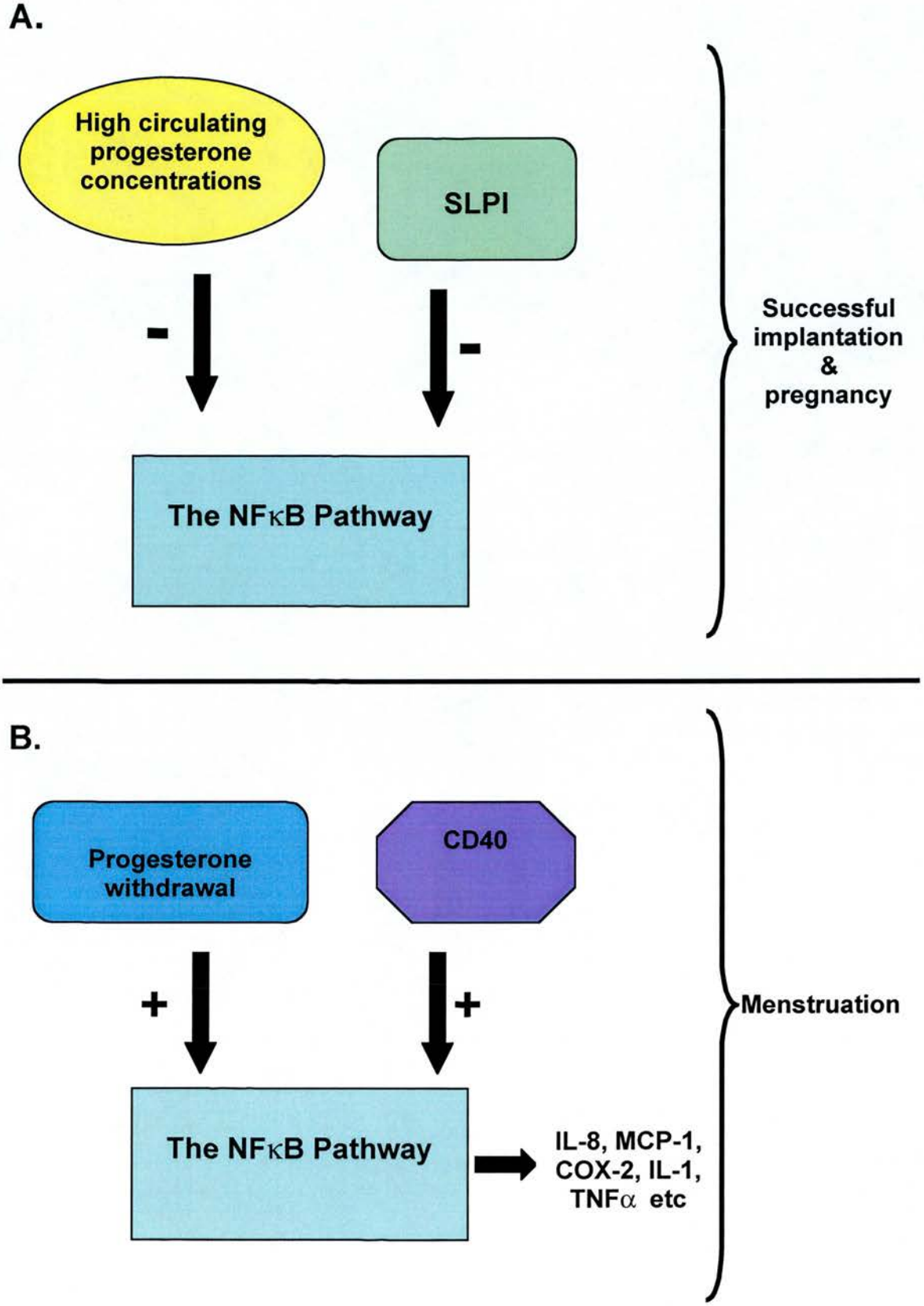
1. To investigate progesterone control of molecules involved in the NFκB pathway.

2. To investigate the expression and regulation of these molecules in human endometrium and first trimester decidua.

3. To investigate the expression of the NFκB activator, CD40 and its ligand, in human endometrium and first trimester decidua.

4. To investigate the expression of the NFκB inhibitor and antibiotic agent, SLPI, in human endometrium and first trimester decidua.

Figure 7: Hypothesized role of NFκB in human endometrium. **(A).** Progesterone will maintain suppression of NFκB during the secretory phase and pregnancy. The presence of anti-inflammatory molecules such as SLPI will contribute to this role. **(B).** Progesterone withdrawal (e.g. prior to menstruation) will result in NF κB activation resulting in the upregulation of chemokines, prostaglandins and MMPs. Pro-inflammatory molecules such as CD40 will enhance the stimulation of the NFκB pathway.


A.
High circulating progesterone concentrations
SLPI
-
-
The NFκB Pathway
Successful implantation & pregnancy
B.
Progesterone withdrawal
CD40
+
+
The NFκB Pathway
IL-8, MCP-1, COX-2, IL-1, TNFα etc
Menstruation

2. General Methods

All materials used in the work described in this thesis are detailed in Appendix 1.

2.1 Human uterine tissue collection

Endometrial biopsies were collected from women (median age 40, range 24-51; no previous pregnancy n=1, previous pregnancy n=60) undergoing gynaecological procedures for benign conditions including hysterectomy (for dysfunctional uterine bleeding and prolapse) and laparoscopic sterilization. Biopsies were collected with an endometrial suction curette (Pipelle, Laboratoire CCD, Paris, France) or alternatively, full thickness endometrial samples were obtained. These included superficial and basal endometrium plus the endometrial-myometrial junction. All women described regular menstrual cycles (25-35 days) and had not received any form of hormonal treatment in the 3 months preceding biopsy. Biopsies were dated according to the patient's reported last menstrual period (LMP) and only included for analysis if the histological dating according to published criteria (Noyes, et al., 1950) and circulating sex steroid concentrations were consistent with the LMP. Serum was separated (by centrifugation at 3000 rpm for 7 min) from venous blood samples collected at the time of biopsy and oestradiol (E_2) and progesterone (P_4) concentrations were measured by radioimmunoassay.

First trimester decidua (8-10 weeks gestation) was obtained from patients (median age 25.5, range 16-38; no previous pregnancy n=12, previous pregnancy n=15) undergoing suction termination of pregnancy. Prior to the termination, biopsies were collected by curettage of the uterine wall away from the site of implantation. Trophoblastic villi were also collected during the procedure. Decidua parietalis (without trophoblast) was confirmed by examination of haemotoxylin and eosin stained sections and cytokeratin immunolocalization confirmed the absence of infiltrating trophoblast cells (see 2.3.7). Additionally, trophoblast samples were included in all quantitative PCR studies that examined mRNA expression in endometrium and decidua (detailed in Chapters 3-5). In all cases trophoblast mRNA expression was similar to, or less than, that of first trimester decidua. This provided

further reassurance that decidual mRNA levels were unlikely to reflect trophoblast contamination. Table 4 details biopsies included in this study.

Tissue samples were collected in RPMI 1640 medium and additionally, biopsies were fixed in 10% neutral buffered formalin (NBF) overnight at 4°C, stored in 70% ethanol and then wax embedded. Also, biopsies to be used in immunohistochemistal studies (section 2.3) were placed in Hanks Balanced Salt Solution supplemented with 0.7% HEPES buffer and microwaved on high power for 12 seconds. After microwaving, biopsies were transferred to cold phosphate buffered saline (PBS) containing 10% sucrose for 15-30 minutes (as described by Slayden, et al. (1995)). Biopsies were then embedded in OCT freezing compound in tin foil moulds, snap frozen in liquid nitrogen and stored at –70°C. Biopsies to be used in PCR studies were immersed in Tri reagent or Ultraspec and then treated as detailed in section 2.2.

Written informed consent was received from all patients prior to biopsy collection and institutional ethical approval had been obtained from Lothian Research Ethics Committee.

Biopsy Type	Day of cycle/weeks pregnant	Number of biopsies	Age of patient (median/range)	Serum oestradiol concentrations (pM; median/range)	Serum progesterone concentrations (nM; median/range)
Menstrual	Days 1-4	5	39 37-43	108.2 57.1-174.0	4.0 2.0-4.4
Early proliferative	Days 5-7	7	36.5 30-48	233.7 43.0-740.0	2.6 1.2-6.2
Mid proliferative	Days 8-10	18	39.5 29-46	426.9 64.3-928.3	3.6 1.5-11.9
Late proliferative	Days 11-13	10	40.5 32-51	363.7 113.4-1446.1	3.0 1.3-5.8
Early secretory	Days 15-18	13	40 24-47	348.0 142.0-931.0	29.8 9.1-108.5
Mid secretory	Days 19-23	7	39 32-42	293.7 120.2-958.3	40.0 15.0-71.3
Late secretory	Days 24-28	9	42 30-48	203.5 54.4-514.7	7.9 2.6-39.4
First trimester decidua	7-11 weeks amenorrhoea	31	25.5 16-38	-	-

Table 4: Details the endometrial and decidual biopsies analysed in this research project. Conventionally, the menstrual cycle is regarded as lasting for 28 days. While this table assumes a 28 day cycle it is acknowledged that the length of the menstrual cycle varies between 25-35 days (Treleor et al., 1967).

2.2 RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR)

2.2.1 RNA extraction

RNA was extracted from tissue and cells using either Ultraspec or Tri reagent. The protocol for use of Tri reagent is detailed below. Tissue was immersed in 1 ml of Tri reagent and then homogenized; cells were harvested and then resuspended in 1ml of Tri reagent. This was followed by a 5 minute incubation period at 4°C. 200µl of choroform was then added and samples were mixed by inversion. After a further 5 minute incubation, samples were centrifuged at 14000 rpm for 15 minutes at 4°C. This allowed the sample to separate into 3 phases. The upper, aqueous phase contained RNA with the remaining phases containing DNA and protein. The aqueous layer was removed to a fresh tube and 500µl of 100% isopropanol was added. After thorough mixing the samples were incubated at –20°C for a minimum of 60 minutes. This was followed by centrifugation at 14000rpm for 10 minutes at 4°C. The resulting supernatant was removed leaving a pellet containing RNA. This was washed in 1 ml of 70% ethanol by brief vortexing followed by centrifugation at 14000 rpm for 5 minute at 4°C. Ethanol was removed and the pellet was resuspended in an RNA storage solution. Samples were stored at –70°C for further use. The protocol for Ultraspec use was identical to this with the exception of one further wash in ethanol.

The optical densities of the RNA samples were measured at 260 and 280nm in order to determine purity and the concentration of RNA present. A 260:280 value of around 1.8 indicates a pure RNA sample. Concentrations of RNA were calculated using the following formula:

260 value x dilution of RNA x 40 = RNA concentration (ng/µl)

For use in RT-PCR all RNA samples were diluted to 100ng/µl.

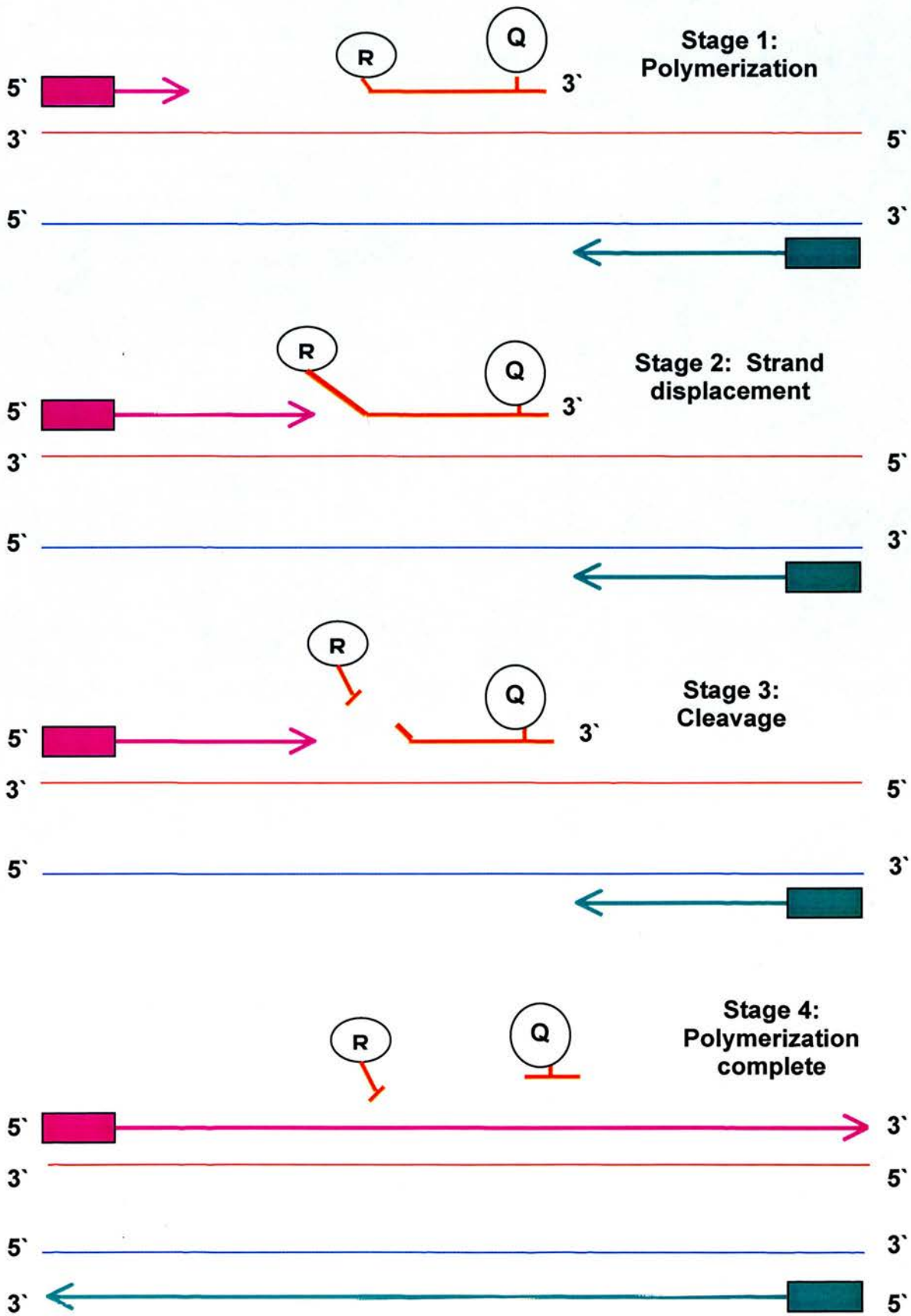
2.2.2 Reverse transcription

All RNA samples were reverse transcribed using random primers with $MgCl_2$ (5.5mM), dNTPs, random hexamers (2.5μM), RNAase inhibitor (0.4U/μl) and multiscribe reverse transcriptase (1.25U/μl). The mix was aliquoted into individual tubes (8μl/tube) and template RNA was added (2μl/tube of 100ng/μl RNA). Mineral oil was added and samples were incubated for 60 minutes at 25°C, 45 minutes at 48°C and then at 95°C for 5 minutes.

2.2.3 Quantitative Polymerase Chain Reaction

The quantitative PCR method used allows the measurement of PCR product via the detection of release of a fluorescent reporter dye. The technique uses forward and reverse primers to the sequence of target DNA along with a probe that recognizes a sequence present between the annealing sequences of the two primers. The probe is labelled with two fluorescent dyes: a 5' reporter dye (all amplicons except 18S use FAM; 6-carboxyfluorescein) and a 3' quencher dye (TAMRA; 6-carboxytetramethylrhodamine). In the intact state the quencher dye, because of its proximity, suppresses the fluorescence of the reporter dye. When the target sequence is amplified during a PCR reaction the probe is cleaved by AmpliTaq Gold (which has Y shape structure and polymerization dependent 5'-3' nuclease activity (Holland, et al., 1991)). This results in the separation of the two dyes so that the quencher dye can no longer suppress the reporter fluorescence. Hence, the fluorescence is increased allowing measurement of the amount of PCR product. (Figure 8). Fluorescence is detected only if the target sequence for the probe is amplified during the reaction thus preventing the detection of non-specific amplification.

Figure 8: Quantitative polymerase chain reaction. Stage 1 shows polymerization of primers and probe to template complementary DNA (cDNA). At this stage flourescence is low as the quencher dye (TAMRA) suppresses the fluorescence of the reporter dye (FAM). As the primer is extended along the template the probe is first displaced (stage 2) and then cleaved by the actions of Taq polymerase (stage 3). This separates the reporter dye from the suppressive effects of the quencher. The fluorescence increases and this allows measurement of the amount of PCR product generated. Polymerization is completed (stage 4). Adapted from Taqman PCR Reagent Kit Protocol.


R
Q
Stage 1:
Polymerization
5`
3`
3`
5`
5`
3`
R
Q
Stage 2: Strand
displacement
5`
3`
3`
5`
5`
3`
R
Q
Stage 3:
Cleavage
5`
3`
3`
5`
5`
3`
R
Q
Stage 4:
Polymerization
complete
5`
3`
3`
5`
5`
3`
3`
5`

Measurement of both ribosomal 18S and specific amplicon levels in the same PCR well is possible as dyes emitting fluorescence of different wavelengths are incorporated into the two probes. 18S levels remain constant (relative to the amount of complementary DNA (cDNA) present) thus allowing changes to specific amplicon levels to be measured. The reporter dye present on the 18S probe is VIC (chemical name unavailable) although initial studies used JOE (2,7-dimethoxy-4,5-dichloro-6-carboxyfluorescein) labelled probe.

Quantitative PCR was used to measure levels of IκBα, IKKα, IKKβ, IKKγ, MEKK-1, NIK, TBK1, IL-8, COX-2, CD40, CD40 ligand, SLPI and PR in the cDNA samples. A reaction mix was made containing Taqman buffer, $MgCl_2$ (5.5mM), dATP (200μM), dCTP (200μM), dGTP (200μM), dUTP (400μM), ribosomal 18S forward and reverse primers and probe (all at 50nM), specific amplicon forward and reverse primers (both 300nM), probe (200nM), AmpErase UNG (0.01U/μl) and Amplitaq Gold DNA polymerase (0.025U/μl). The mixture was then aliquoted into separate tubes for each cDNA sample. 1μl/replicate of cDNA was added to each tube. After mixing, 23μl of sample were added to the wells on a PCR plate. Each sample was added in triplicate Wells were sealed with optical caps and the PCR reaction run on ABI Prism 7700 using standard conditions.

Three steps were taken to ensure that there was not genomic DNA contamination of PCR samples. A no template control (containing water) and an RNA sample which had not undergone reverse transcription were included in triplicate in each PCR run. Additionally, β-actin levels were measured in all RNA samples (without reverse transcription). Any sample which had a β-actin measurement below an arbitrary level of 27 (i.e. 3 standard deviations from the mean of all samples) was excluded. Therefore, two endometrial samples were excluded from analysis. Figure 9 shows the β-actin measurements of all RNA samples.

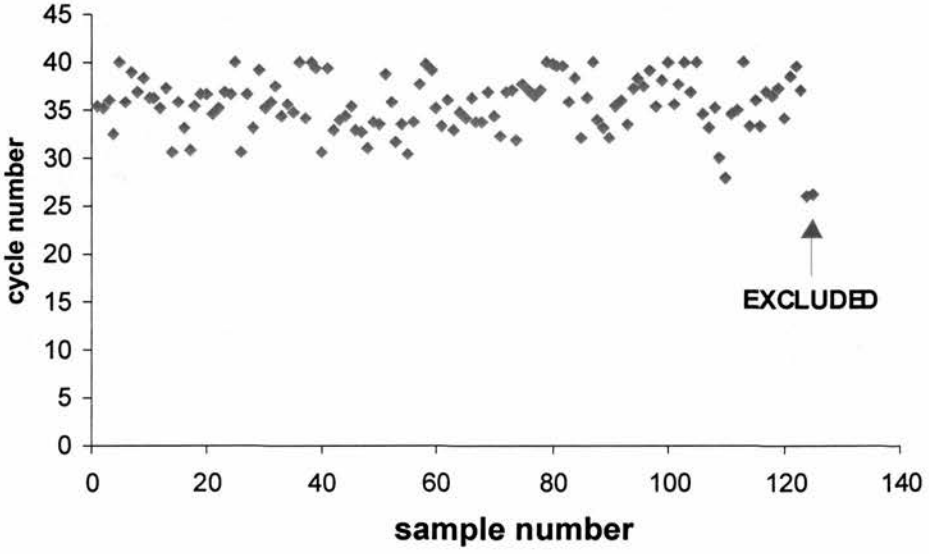


Figure 9: β-actin levels in all RNA samples that were subsequently used for RT-PCR. The PCR cycle number at which fluorescence release reached a threshold level is shown for each sample. Any sample with a β-actin measurement below 27 (indicating likelihood of DNA contamination) was excluded from the study. This meant that two samples were excluded from analysis (shown by red arrow on graph).

The linearity of the response to specific cDNA was determined by serial dilution. The concentrations of primers and probes were diluted up to 64 times using a standard pool of RNA. The log of ng total RNA was plotted against ΔCt units and the gradients of the lines through these points were less than 0.1. Figure 10 shows a representative validation plot. Within assay variation of the PCR measurement of specific amplicon in cDNA was calculated from six replicates (relative standard deviation; see Table 5).

Primers and probes were designed (using the PRIMER express program) to ensure successful hybridization with the template cDNA and efficient amplification. BLAST (Basic Local Alignment Search Tool) searches were used to determine the presence of sequences in the scientific databases that are similar to those amplified by each set of primers. The results of these searches showed that, in all cases, the primer and probe sets used were unlikely to amplify an inappropriate template. The results of BLAST searches give an expect value (E) which shows the number of hits expected to be found by chance when searching a database. If the E value is equal to 1 this indicates that 1 sequence is expected to match that of interest by chance. The E values along with the sequences of primers and probes are shown in Table 5.

PCR data was analyzed using the formula $2^{-\Delta\Delta CT}$ (CT is the cycle number at which the PCR signal crosses a threshold; ΔCT is the difference between the CT values for the specific amplicon and 18S; ΔΔCT relates the ΔCT value of each sample to that of an internal control). This normalizes the amount of target mRNA to the amount of 18S RNA and then, relates this value to that of an internal control.

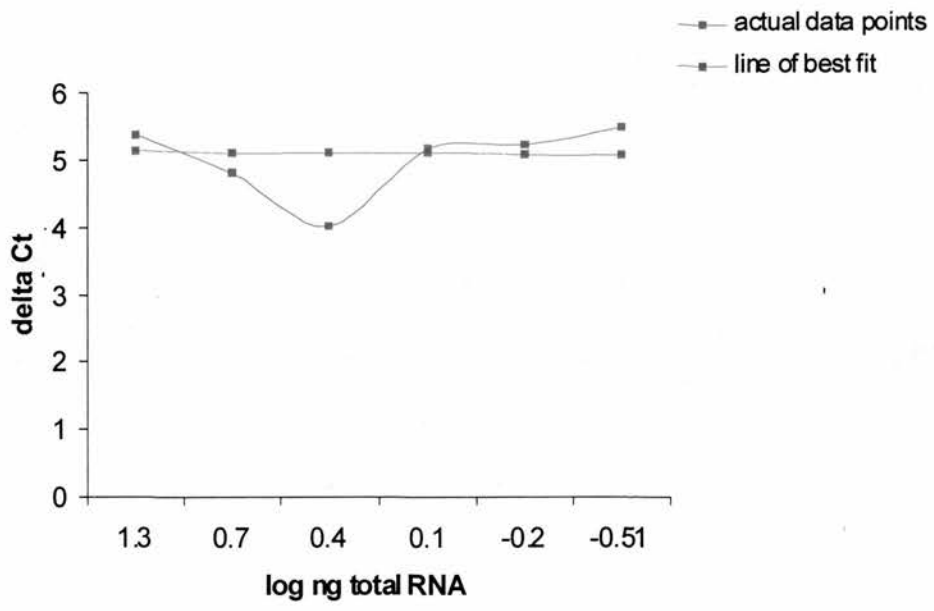


Figure 10: Validation of secretory leukocyte protease inhibitor (SLPI) PCR primers and probe. Validation was performed for each set of primers and probes used in this research project and this figure is representative of these experiments. The delta Ct values (difference between the CT values for SLPI and 18S; CT is cycle number at which fluorescence release reaches a threshold value) are plotted against the log ng total RNA present. For successful validation the gradient of the line of best fit must be less than 0.1. In this case the equation of the line was: $y=0.029x + 5.103$.

Table 5: Details of PCR primers and probes utilized in this project. Within assay precision values and BLAST search E values for each set of primers and probe are also shown.

* Standard set of primers and probe designed by PE Biosystems.

** Standard set of primers and probe designed by PE Biosystems. These sequences relate to the JOE labelled 18S probe. Sequences for the VIC labelled probe are currently unavailable. Within assay precision cannot be provided for 18S measurements as it is not possible to correct this data relative to the amount of cDNA present.

Amplicon	Accession number (mRNA sequence)	Forward primer	Reverse primer	Probe	E value from BLAST search	Within assay precision
*β-actin	X00351	TCACCCACAC TGTGCCCATC TACGA	CAGCGGAAC CGCTCATTGC CAATGG	ATGCCCCCCC CATGCCATCC TGCGT	e^{-160}	1.0%
CD40	X60592	CACTGCCACC AGCACAAAT ACT	TCTGTTTCTG AGGTGCCCTT CT	CGACCCCAAC CTAGGGCTTC GG	$5e^{-34}$	8.1%
CD40 ligand	Z15017	GTGCTTCGGT GTTTGTCAAT GT	CAGCCTGCAA GGTGACACTG T	ACTGATCCAA GTGAGCCATG G	$2e^{-53}$	7.3%
COX-2	M90100	GTGTTGACAT CCAGATCACA TTTGA	GAGAAGGCT TCCCAGCTTT TGTA	TGACAGTCCA CCAACTTACA ATGCTGACTA TGG	$3e^{-39}$	2.0%

Amplicon	Accession number (mRNA sequence)	Forward primer	Reverse primer	Probe	E value from BLAST search	Within assay precision
IKKα	AF009225	CATGGTATTT GAATGTATTG CTGGATA	CTTTGGATCC TTCTTCTTAA TCTTCTCAT	AGGCCTTTTT TGCATCATCT GCAGCC	$9e^{-46}$	1.1%
IKKβ	AF080158	GGAAGCCCG GATAGCATG A	TTCTTGGCTG GCTCAGGTAA G	CTCTCGACTT AGCCAGCCTG GGCA	$6e^{-50}$	0.6%
IKKγ	AF074382	CAGCAGATG GCTGAGGAC AAG	TCCTTAGTGG CAGCCTCCAA	TGAAAGCCC AGGTGACGTC CTTGC	$6e^{-50}$	1.0%

Amplicon	Accession number (mRNA sequence)	Forward primer	Reverse primer	Probe	E value from BLAST search	Within assay precision
IL-8	M26383	CTGGCCGTGG CTCTCTTG	TTAGCACTCC TTGGCAAAAC TG	CCTTCCTGAT TTCTGCAGCT CTGTGTGAA	$9e^{-36}$	4.0%
IκBα	M69043	TTGGGTGCTG ATGTCAATGC	AGGTCCACTG CGAGGTGAA G	CAGTCCGGCC ATTACAGGGC TCCT	$2e^{-30}$	0.5%
MEKK-1	AF042838	TGCGGGCCA GACTGTACTT ACT	TGCAGTTCTG AGGCCCAAT AA	CAGCAGATA GGGCCTAACT CTTTCCTGAT TGG	$5e^{-54}$	1.8%
NIK	Y10256	GAAGAAACA GAGCTCCGTC TACAAG	CATTCAGGAT CTCCCACTTT CC	CACAGGGCTC TTCTCCACGG CCT	$2e^{-37}$	0.6%

Amplicon	Accession number (mRNA sequence)	Forward primer	Reverse primer	Probe	E value from BLAST search	Within assay precision
Progesterone receptor (A and B isoforms)	M15716	CAGTGGGCGTTCCAAATGA	TGGTGGAATCAACTGTATGTCTTGA	AGCCAAGCCCTAAGCCAGAGATTCACTTT	$3e^{-39}$	0.8%
SLPI	AF114471	GCATCAAATGCCTGGATCCT	GCATCAAACATTGGCCATAAGTC	TGACACCCCAAACCCAACAAGGAGG	$2e^{-43}$	9.7%
TBK1	AF191838	GCAGAACCGCACCACTGTTA	GATCTGGGCACCTTGTAAAATAAAATA	TGCCACTTTATCCTTCTCAAAAAAATTGTTATCCCAG	$4e^{-29}$	1.2%
****Ribosomal 18S**	X03205 (ribosomal RNA)	CGGCTACCACATCCAAGGAA	GCTGGAATTACCGCGGCT	TGCTGGCACCAGACTTGCCCTC	e^{-101}	N/A

2.3 Immunohistochemistry

Immunohistochemistry was performed in order to detect NIK, IKKα, CD40, Thy-1, CD1a, SLPI and cytokeratin proteins in tissue biopsies. All protocols were optimized to determine appropriate conditions for maximum immunostaining. Protocols are summarized in Table 6.

Throughout this thesis the term 'perivascular' is used to describe the presence of cells and proteins in the region associated with the endometrial vasculature. Endothelial, fibroblast and smooth muscle cells are present in this area. This term has been used to describe similar cell/protein localization in previous studies (Critchley, et al., 1999; Jones, et al., 1997; Wang, et al., 1998).

EPITOPE	FROZEN	PARAFFIN		NON-IMMUNE BLOCK SERUM	DILUTION OF 1^{O}	NEGATIVE CONTROL	2^{O} USED	TIME WITH 2^{O} AND 3^{O}
		no MW	MW					
NIK			✓	goat	1:200	rabbit IgG	goat anti rabbit	60 min
IKKα			✓	goat	1:800	rabbit IgG	goat anti rabbit	40 min
CD40	✓			horse	1:4	mouse IgG	horse anti mouse	60 min
Thy-1	✓			horse	1:10	mouse IgG	horse anti mouse	60 min
CD1a			✓	horse	1:50	mouse IgG	horse anti mouse	60 min
SLPI		✓		horse	1:400	goat IgG	horse anti goat	60 min
cytokeratin		✓		horse	1:60	mouse IgG	horse anti mouse	60 min

Table 6: Details protocols used for immunohistochemical investigation of protein expression in reproductive tissues. MW=microwave antigen retrieval step. All incubations with primary antibodies were overnight at 4°C with the exception of the cytokeratin antibody (1 hour at 37°C).

2.3.1 NIK

NIK immunohistochemistry was performed on paraffin sections (5μm). These were dewaxed in histoclear and rehydrated in descending grades of alcohol. After washing in PBS a microwave antigen retrieval step was performed. Sections were heated in 0.01M sodium citrate (pH 6) on full power for 10 minutes and were then incubated for 20 minutes in the oven. After further washing in PBS endogenous peroxidase activity was blocked with 3% hydrogen peroxide in distilled water for 10 min at room temperature. Sections were washed for 10 min in PBS and then diluted normal goat serum was applied to all tissue sections for 20 min in a humidified chamber at room temperature. Tissue sections were incubated overnight at 4°C with 50μl of rabbit anti-NIK antibody (1:200 dilution in goat serum). The primary antibody was substituted with an equimolar concentration of rabbit immunoglobulin (IgG) in negative control sections. After the primary antibody incubation, sections were washed between each stage for 10 min in PBS + Tween. Sections were incubated with biotinylated goat-anti rabbit IgG and then with an avidin-biotin peroxidase detection system (ABC; both for 60 min at room temperature). The peroxidase substrate diaminobenzidine, which forms a brown precipitate on contact with the antigen-antibody complex, was used to identify positive staining. Sections were counterstained with Harris's haemotoxylin, dehydrated in ascending grades of alcohol and mounted from xylene in Pertex.

2.3.2 IKKα

This protocol was identical to that for NIK immunolocalization with the exception that the primary antibody was anti-IKKα (diluted 1:800 in goat serum) and incubations with the secondary antibody and ABC were for 40 minutes only.

2.3.3 CD40

CD40 immunostaining was performed on frozen tissue sections (5μm). These were lightly fixed in NBF for 10 min at room temperature. After washing in PBS an endogenous peroxidase block was carried out and the remainder of the protocol was similar to that of NIK (section 2.3.1). The non-immune block used normal horse serum. The primary antibody was mouse anti-CD40 (G28-5; diluted 1:4 in horse serum) and equimolar mouse IgG was applied to negative control sections (overnight at 4°C). Sections were sequentially incubated with biotinylated horse-anti mouse IgG and ABC (both 60 minutes, room temperature). The protocol was completed as for NIK.

2.3.4 Thy-1

Thy-1, a molecule expressed on fibroblasts, was immunolocalized using an identical protocol to that for CD40 with the exception that the primary antibody was for Thy-1 (1:10 dilution in horse serum). The negative control sections were incubated with mouse IgG at a concentration of 1μg/ml. The Thy-1 antibody was in a culture supernatant and so, the antibody concentration could not be determined. Hence, the concentration of mouse IgG was not matched to that of anti-Thy-1.

2.3.5 CD1a

Immunolocalization of the dendritic cell marker, CD1a, was performed on paraffin sections and the protocol was similar to that of NIK. A microwave antigen retrieval step was necessary prior to the endogenous peroxide block. The non-immune block used normal horse serum and the primary antibody was anti-CD1a (1:50 dilution in horse serum). The concentration of antibody was unknown so the mouse IgG could not be concentration matched. It was used at a concentration of 2μg/ml. Sections were sequentially incubated with biotinylated horse-anti mouse IgG and ABC (both 60 minutes, room temperature). The protocol was then completed as described for NIK.

2.3.6 SLPI

SLPI immunohistochemistry was performed on paraffin sections. After this stage the protocol was similar to that of NIK. There was no antigen retrieval step. The primary antibody was goat anti-SLPI (1:400 in horse serum) and negative control sections were incubated with equimolar concentrations of goat IgG. After overnight incubation at 4°C, the sections were treated with biotinylated horse-anti goat IgG and then, ABC (both for 60 min at room temperature). The protocol was then completed as above.

2.3.7 Cytokeratin

Cytokeratin immunolocalization was performed to confirm the absence of infiltrating trophoblast cells in decidual biopsies. Cytokeratin is present in endometrial epithelial cells but any additional immunoreactivity indicates the presence of trophoblast cells. Any biopsies found to have such contamination were excluded from the study.

Immunolocalization of cytokeratin used paraffin sections and the protocol was similar to that of NIK. However, no antigen retrieval step was necessary. The primary antibody was mouse anti-cytokeratin (1:60 in horse serum). Negative control sections were incubated with equimolar concentrations of mouse IgG. It should be noted that incubation with the primary antibody was for 1 hour at 37°C. Sections were then treated with biotinylated horse-anti mouse IgG and ABC (both for 60 min at room temperature). The protocol was completed as described above.

2.3.8 Scoring of immunohistochemistry

Location and intensity of NIK and IKKα immunostaining was measured using a semi-quantitative scoring system. A score of 0 indicated no immunoreactivity; 1 faint immunoreactivity; 2 strong immunoreactivity and 3 very strong immunoreactivity. Sections were scored by two observers (blind to the stage of menstrual cycle). This scoring system is a standard method used in previous studies (Critchley, et al., 1998b; Jones, et al., 1997; Wang, et al., 1998).

2.4 *In vitro* culture

2.4.1 Tissue Culture

Tissue was cultured on sterilized polypropylene capillary matting in RPMI 1640 supplemented with 10% fetal calf serum, penicillin (50μg/ml), streptomycin (50μg/ml) and gentamycin (5μg/ml; complete medium). Endometrium and decidua were cultured in the presence of oestradiol (10^{-8}M) and any additional treatments (detailed in chapters 3-6) were added. Tissue was incubated for 24 hours and then culture medium was removed for subsequent inclusion in enzyme linked immunosorbent assays (ELISAs). Tissue was weighed after incubation and all assay measurements were corrected for weight. Tissue that was to be collected for RNA extraction was immersed in Tri reagent immediately after culture and processed as detailed in section 2.2.

2.4.2 Cell Culture

HeLa and Ishikawa cells were cultured in 24 well cultured plates; T47D and MFE cells were cultured in 25cm^3 culture flasks. All cells were cultured at a concentration of $2x10^5$cells/ml in complete medium. After passaging cells were incubated for 24 hours to allow adherence to the well/flask. Treatments were added and cells were incubated for an appropriate time (detailed in chapters 3-6). Culture supernatants were then removed for subsequent inclusion in ELISAs. In the event that cells were

collected for RNA extraction (see section 2.2), Tri reagent was added directly to culture wells and then harvested. Cells in culture flasks were trypsinized, centrifuged to form a pellet (2000 rpm, 3 min) and then resuspended in Tri reagent.

2.5 Enzyme linked immunosorbent assays (ELISA)

IL-8 and SLPI protein concentrations in culture supernatants were determined by ELISA. The protocol for the IL-8 ELISA has been previously described (Denison, et al., 1997).

2.5.1 IL-8 ELISA

Plates were passively coated overnight at 4°C with 100µl/well of capture antibody (4µg/ml). After incubation, plates were washed in water and 100µl/well of blocking solution was added. After 30 min at room temperature the solution was flicked out and plates were air dried and stored at 4°C. Prior to use, plates were washed in water. Standards were diluted in ELISA buffer (top standard 500pg/ml). 100µl/well of standard or sample was added. Plates were sealed and incubated overnight at 4°C. Plates were washed 4 times in wash buffer and then detection antibody (50ng/ml) was added at 100µl/well. Incubation was for 90 min on a plate shaker at room temperature. Plates were washed as above. 100µl/well of streptavidin peroxidase (1:1000 dilution) was added and plates were incubated for 20 min on a plate shaker at room temperature. Plates were washed as above. 200µl/well of substrate was added. Plates were left for 20 min, quenched with 50µl/well of 2N sulphuric acid and then read on a plate reader at 450nm.

2.5.2 SLPI ELISA

Assay plates (96 well) were coated with 0.025μg/ml recombinant SLPI in PBS and 1% 400mM carbonate buffer. 100μl was added to each well. Plates were left for 60 min at room temperature. Blocking was carried out with 400μ l/well blocking/protecting solution for 30 min. Plates were washed (wash buffer). 150μl of standard/sample and 50μl of anti-SLPI (2μg/ml; diluted in ELISA buffer) were added to each well. A non specific binding well (200μl buffer only) and two B_0 wells (150μl buffer; 50μl anti-SLPI) were included on each plate. Standards were added in triplicate and their concentration range was from 0.098-50ng/ml. Incubation was on a plate shaker at room temperature for 2 hours. Plates were washed. 100μl/well of peroxidase labeled anti-sheep/goat IgG Fab fragments raised in donkey (1:1000 dilution of stock in ELISA buffer) was added. Incubation was on a shaker as before. Plates were washed. 200μl substrate was added to each well. After 2-10 minutes wells were quenched with 50μl/well 2N sulphuric acid. Plates were read in a plate reader at 450nm.

2.6 Statistical analysis

All statistical analysis performed in this research project used Statview 3.0. Statistical tests used are described in individual chapters. Throughout this thesis statistical differences are indicated on graphs by the use of letters (shown above the relevant bars). For example, in Figure 14 the letter 'a' indicates that IκBα mRNA expression in perimenstrual endometrium is significantly different to that in the proliferative phase. P values relating to the letters used are shown in the top right of each figure.

3. The NFκB pathway in human endometrium and first trimester decidua

3.1 Introduction

The endometrium is a major target tissue of progesterone and the hormone is crucial to successful implantation and pregnancy. Its presence during the secretory phase of the menstrual cycle is vital to the development of the endometrium in preparation for implantation. For example, as a consequence of progesterone production, glandular secretory activity ensues, pinopodes develop and production of chemokines such as MCP-1 and IL-8 is suppressed (Jones, et al., 1997; Martel, et al., 1989). The presence of the hormone is also required for the stromal decidualization that occurs in the late secretory phase and further, in early pregnancy. Equally, progesterone is critical to the maintenance of pregnancy once established. It causes immunosuppression at a time when inflammatory and immune events would be detrimental to the development of the fetus (Siiteri and Stites, 1982). This occurs via several mechanisms, some of which may be indirect. For instance, suppression of inflammatory mediator expression and modulation of T cell activity may be induced by cytokine release from endometrial cells that possess the progesterone receptor.

The withdrawal of progesterone at the end of the menstrual cycle is a crucial event in the initiation of the proinflammatory events associated with menstruation. Endothelins and prostaglandins mediate vasoconstrictive events (Cameron, 1992) and upregulation of proinflammatory molecules such as IL-8, MCP-1 and COX-2 occurs upon progesterone withdrawal (Critchley, et al., 1999; Jones, et al., 1997). Leukocytes infiltrate the tissue (Jeziorska, et al., 1995; Poropatich, et al., 1987) and tissue degradation occurs under the influence of MMPs (Lockwood, et al., 1998; Salamonsen and Woolley, 1999).

Although progesterone production and its subsequent withdrawal are the key processes involved in implantation/pregnancy and menstruation the precise molecular mechanisms by which it mediates its actions are unclear. The NFκB pathway has been implicated in the production of many inflammatory molecules e.g. IL-8, MMPs, COX-2 (Adcock, et al., 1997; Mauviel, et al., 1992; Vincenti, et al., 1998) and several such molecules are involved in endometrial function. However, there is

little data regarding the role of NFκB in endometrium although it has been reported to be involved in the upregulation of LIF and IL-6 associated with implantation (Laird, et al., 2000). As detailed previously, progesterone (and other steroid hormones) suppresses the NFκB pathway at several levels and it has been suggested that this may be involved in reproductive function (van der Burg and van der Saag., 1996). Cross-talk between these pathways in endometrium is likely. Suppression of NFκB by progesterone would be consistent with the immunosuppressive effects of progesterone that occur during the secretory phase of the cycle and in pregnancy. In contrast, progesterone withdrawal premenstrually may release inhibition of the pathway and would allow activation of NFκB. This may contribute to the inflammatory events surrounding menstruation.

This chapter details the expression and possible regulation of NFκB pathway intermediates in human endometrium and first trimester decidua. Additionally, the effects of NFκB inhibition on inflammatory molecule expression by endometrial epithelial cells are discussed.

3.2 Methods

3.2.1 Human uterine tissue collection

Endometrial (n=32), decidual (n=6) and trophoblast (n=5) samples were collected as detailed in section 2.1. Biopsies utilized in this chapter are detailed in Table 7.

Stage of cycle/tissue biopsy type	Number of biopsies
Menstrual	3
Early proliferative	3
Mid proliferative	5
Late proliferative	4
Early secretory	7
Mid secretory	6
Late secretory	4
First trimester decidua	6
Trophoblast villi	5

Table 7: Details biopsies used in the studies presented in Chapter 3.

Biopsies used in the RT-PCR studies (detailed below) were treated as three groups: perimenstrual, proliferative and secretory phases. The term 'perimenstrual' is used to describe biopsies from the premenstrual and menstrual phases of the cycle (Figure 11). Although the histological appearance of premenstrual and menstrual tissue differs, the circulating levels of oestradiol and progesterone at these times are very similar (circulating sex steroid concentrations are shown in Figure 12 & 13). Initiation of menstruation results from the withdrawal of progesterone. Therefore, investigation of biopsies from the time immediately prior to, and during, menstruation is the most relevant to studies of the mechanisms involved in menstruation.

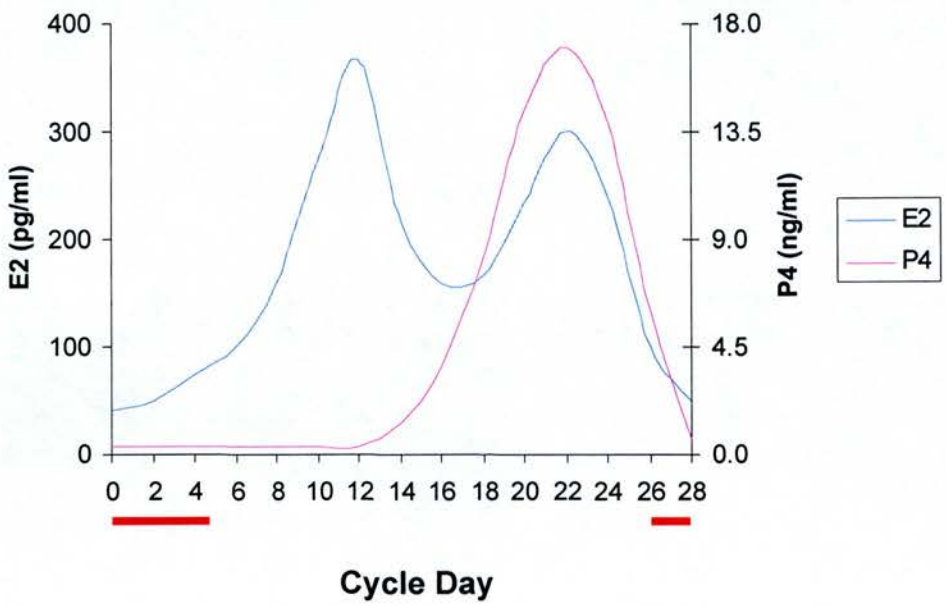


Figure 11: The perimenstrual phase. In this thesis the term 'perimenstrual' is used to describe endometrium from the premenstrual and menstrual phases of the cycle. At these times circulating levels of both oestradiol (E2) and progesterone (P4) are low. The perimenstrual phase lasts from approximately day 26 to day 5 of the menstrual cycle and is highlighted by the red line. Data regarding oestradiol and progesterone levels are adapted from Marshall and Odell, 1989.

Figure 12: Serum oestradiol (E2) concentrations relating to endometrial biopsies collected during the perimenstrual (peri), proliferative (prol) and secretory (sec) phases of the menstrual cycle. 'n' numbers are shown above bars. Paired letters indicate statistical significance (a:$P<0.05$).

Figure 13: Serum progesterone (P4) concentrations relating to endometrial biopsies collected during the perimenstrual (peri), proliferative (prol) and secretory (sec) phases of the menstrual cycle. 'n' numbers are shown above the bars. Paired letters indicate statistical significance (a and b:$P<0.01$).

Figure 12

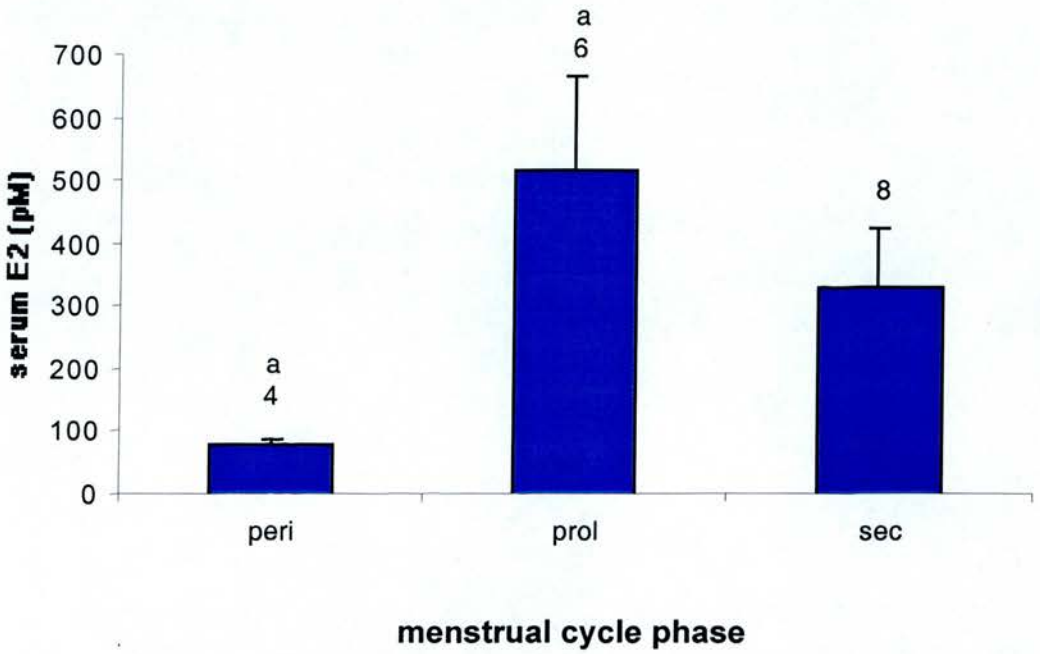
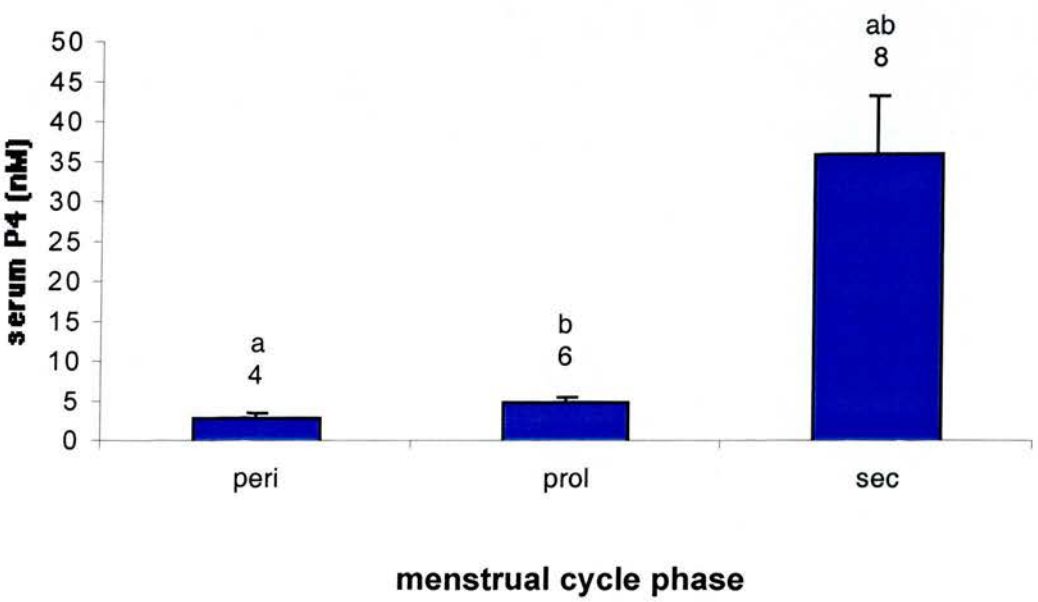


Figure 13



3.2.2 Uterine tissue analyses

3.2.2a RNA extraction and RT-PCR

RNA was extracted from endometrial (n=14), decidual (n=6) and trophoblast (n=5) biopsies and cDNA was prepared as detailed in general materials and methods, 2.2.1 and 2.2.2.

PCR reactions were performed on these cDNA samples (see 2.2.3). Expression of mRNA for the following NFκB pathway intermediates was measured: IκBα, IKKα, IKKβ, IKKγ, MEKK1, NIK and TBK1.

3.2.2b Immunohistochemistry

IKKα and NIK proteins were localized in endometrium (n=18) and decidua (n=4) using the protocols described in 2.3.1 and 2.3.2. Location and intensity of protein immunolocalization was subsequently scored as detailed in the material and methods (section 2.3.8).

3.2.3 *In vitro* cell culture studies (cell line)

3.2.3a Cell culture

MFE cells (endometrial epithelial cell line) were cultured as described in 2.4.2. Treatments were as described in Table 8.

Treatment	Concentration used	Incubation time
Control	N/A	0 and 24 hours
IL-1	1ng/ml	24 hours
MG132	3μM	
MG132 + IL-1	3μM + 1ng/ml	
SN50	5μg/ml	
SN50 + IL-1	5μg/ml + 1ng/ml	

Table 8: Details treatment of the MFE endometrial epithelial cell line.

MG132 and SN50 both inhibit the activation of NFκB. MG132 is a peptide aldehyde proteasome inhibitor. Inhibition of proteasome activity prevents degradation of IκBα causing retention of NFκB in the cytoplasm (Bush, et al., 1997). SN50 is a cell permeable peptide which carries the nuclear localization sequence of the NFκB p50 subunit and so, prevents the translocation of NFκB into the nucleus (Lin, et al., 1995).

3.2.3b RNA extraction and RT-PCR

After incubation RNA was extracted and cDNA prepared. COX-2, IL-8 and IκBα mRNA levels were measured in these cDNA samples by quantitative PCR (see 2.2).

3.2.3c IL-8 ELISA

Culture supernatants from the above cell culture experiments were collected for subsequent inclusion in IL-8 ELISAs (see 2.5.1).

3.2.4 Statistical analysis

PCR (tissue only) results were analyzed using analysis of variance (ANOVA) (Statview 3.0). Individual differences were assigned using Fisher's protected least squares differences (PLSD) test. Immunohistochemical semi-quantitative scoring results were analysed using non-parametric statistics. Differences between immunoreactivity in different endometrial compartments (e.g. glands, stroma) throughout the menstrual cycle were assessed using the Kruskall-Wallis method. Variations in immunoreactivity in equivalent compartments in the functionalis and basalis regions of endometrium from the same tissue sections (e.g. glands in functionalis vs glands in basalis) were determined using the Wilcoxin rank test.

3.3 Results

3.3.1 Uterine tissue analyses

3.3.1a Progesterone withdrawal (menstruation) and high progesterone levels (pregnancy) have differential effects on NFκB pathway intermediates mRNA levels

IκBα mRNA levels are increased in perimenstrual endometrium relative to other cycle stages (Figure 14; $P<0.01$). In addition, a trend towards increased IκBα levels in secretory relative to proliferative endometrium is noted. There is a further increase in first trimester decidua and this is significantly higher than levels in the proliferative phase (Figure 14; $P<0.05$).

The molecules involved in activation of NFκB are found to be differentially expressed in decidua. NIK expression is increased in first trimester decidua compared to endometrium from all cycle stages (Figure 15; $P<0.001$). In contrast, MEKK1 mRNA levels are decreased in first trimester decidua relative to endometrium (Figure 17; decidua/perimenstrual and decidua/secretory $P<0.05$; decidua/proliferative $P<0.01$).

Amounts of IKKα mRNA are found to be increased in first trimester decidua (Figure 16; decidua/perimenstrual $P<0.05$; decidua/secretory $P<0.01$) while IKKβ mRNA levels are decreased in decidua relative to endometrium from the perimenstrual phase (Figure 18; $P<0.05$). Messenger RNA expression of the scaffolding protein, IKKγ, is decreased in first trimester decidua relative to endometrium from the secretory phase of the cycle (Figure 19; $P<0.05$).

Messenger RNA for TBK1, a kinase with homology to an inducible IKK, is expressed at higher levels in endometrium from the perimenstrual phase when compared to biopsies from the secretory phase (Figure 20; $P<0.05$).

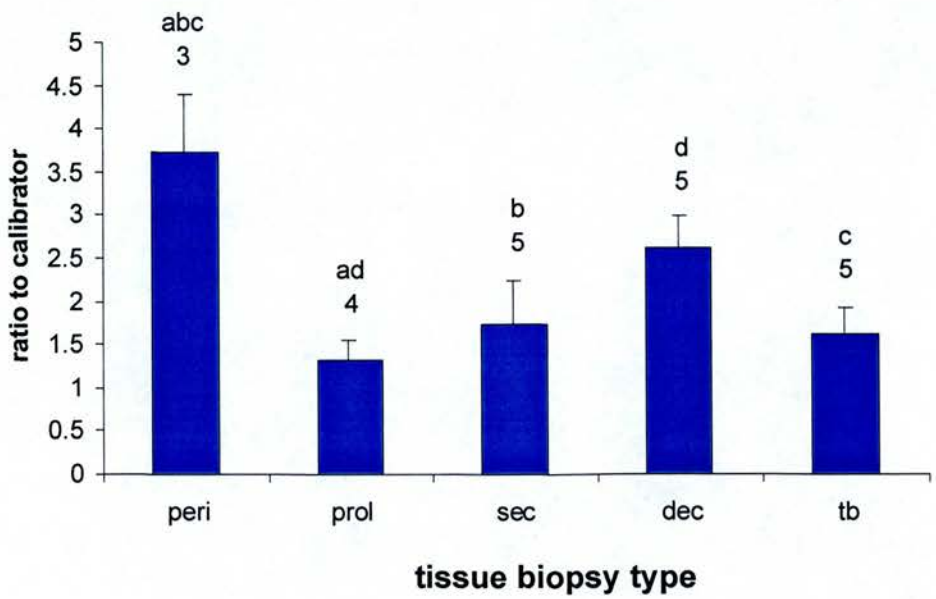


Figure 14: IκBα mRNA expression in perimenstrual (peri), proliferative (prol) and secretory (sec) endometrium, decidua (dec) and trophoblast (tb). All sample PCR measurements are related to an internal control (proliferative endometrial sample) and these ratio are presented in the figure (y axis = ratio to calibrator). 'n' numbers are shown above bars. Paired letters indicate statistical significance (a, b and c:$P<0.01$; d:$P<0.05$).

Figure 15

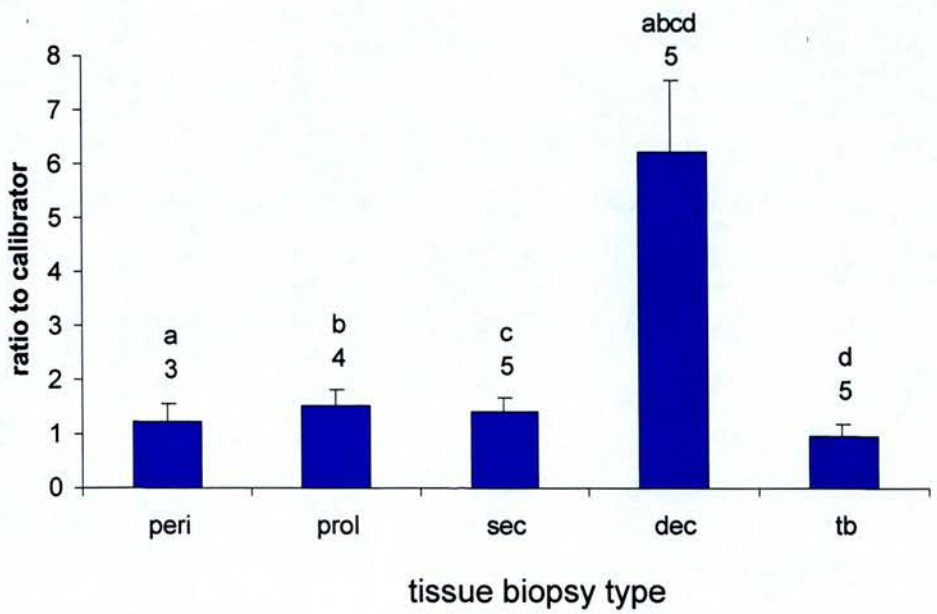


Figure 16

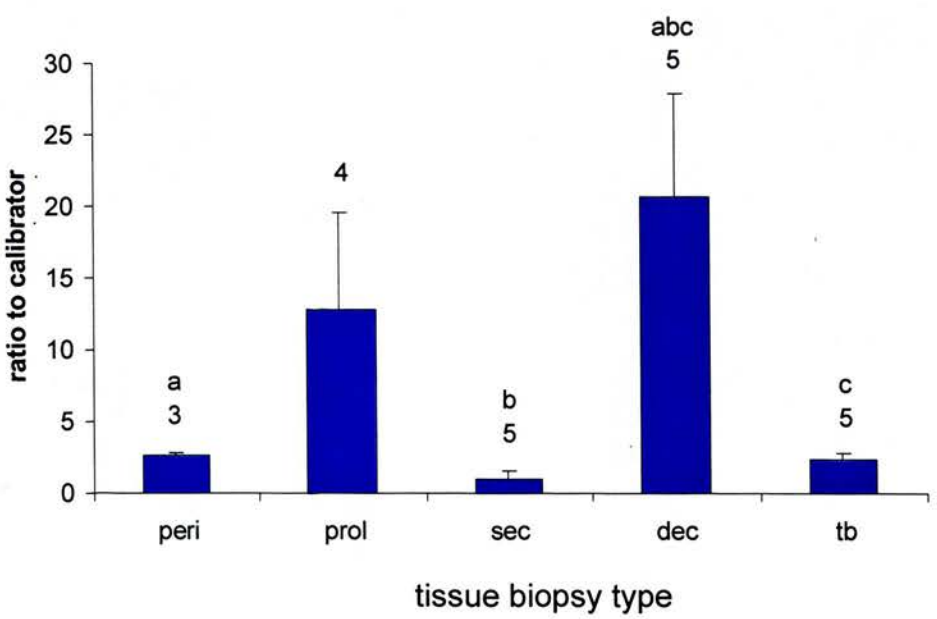


Figure 17: MEKK1 expression in perimenstrual (peri), proliferative (prol) and secretory (sec) endometrium, decidua (dec) and trophoblast (tb). All sample PCR measurements are related to an internal control (proliferative endometrial sample) and these ratio are presented in the figure (y axis = ratio to calibrator). 'n' numbers are shown above bars. Paired letters indicate statistical significance (a and e:$P<0.05$; b, c, d and f:$P<0.01$).

Figure 18: IKKβ mRNA expression in perimenstrual (peri), proliferative (prol) and secretory (sec) endometrium, decidua (dec) and trophoblast (tb). All sample PCR measurements are related to an internal control (proliferative endometrial sample) and these ratio are presented in the figure (y axis = ratio to calibrator). 'n' numbers are shown above bars. Paired letters indicate statistical significance (a:$P<0.05$; b, c and d:$P<0.01$).

Figure 17

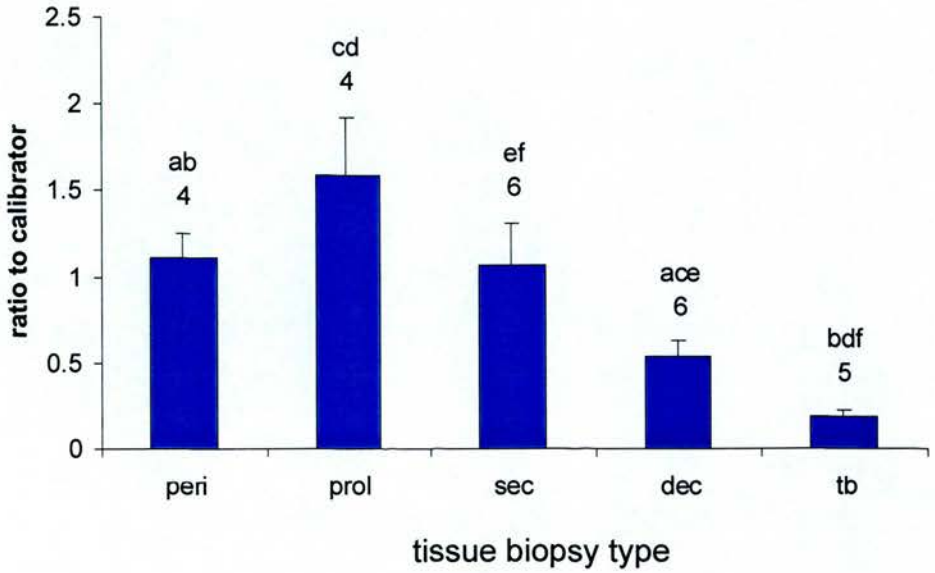


Figure 18

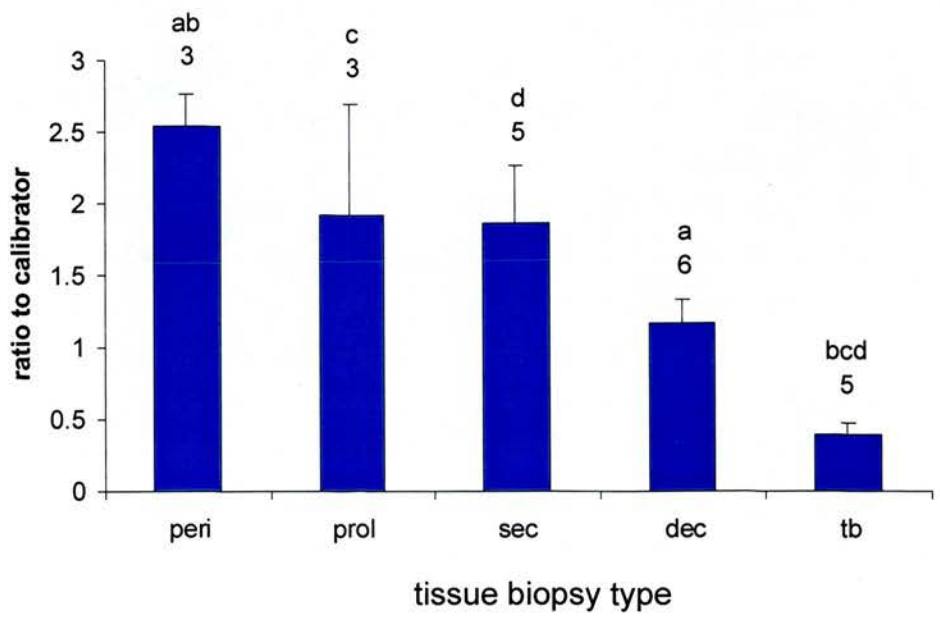


Figure 19

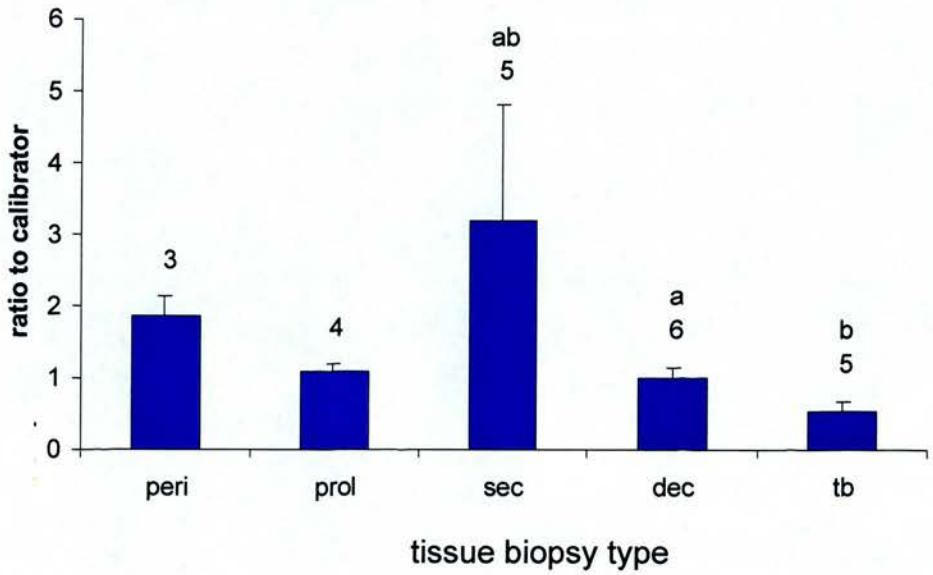
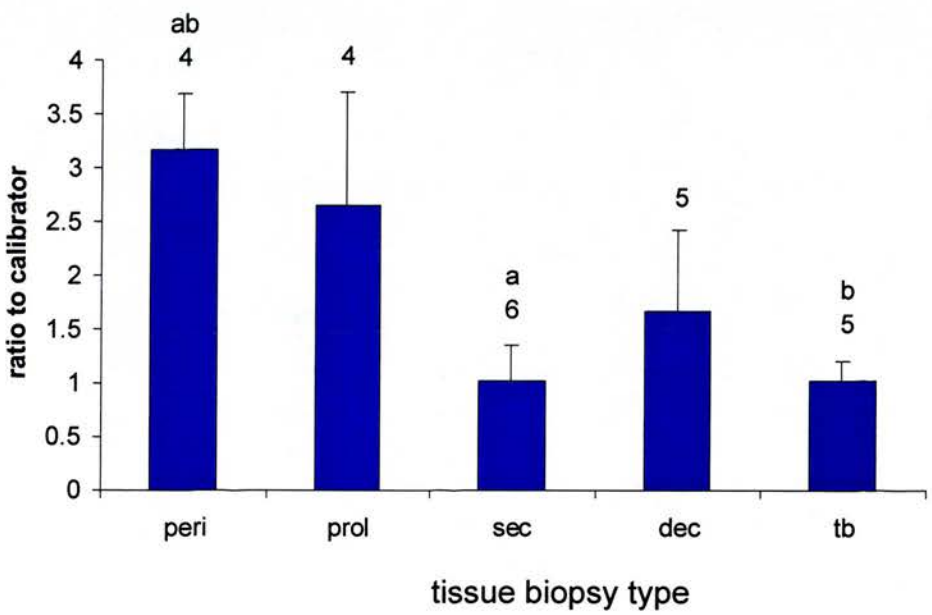


Figure 20



3.3.1b Localization of NIK and IKKα in human endometrium and first trimester decidua

Both NIK (Figure 21 a&b) and IKKα (Figure 22 a&b) proteins were present predominantly in the glandular epithelial cells of endometrium from all stages of the menstrual cycle. Endothelial immunostaining was also observed in some blood vessels. Little stromal staining was present and was only observed in the stroma adjacent to the luminal epithelium.

No significant changes to immunolocalization of the proteins were detected during the menstrual cycle. There was a trend towards decreased IKKα stromal immunoreactivity in the late secretory phase but this was likely to be due to the sloughing of the surface endometrium associated with menstruation. A trend towards increased expression of IKKα in the functionalis layer of the endometrium was also observed. This was apparent for all compartments at all stages of the cycle but was only significant for the stroma in the proliferative phase ($P<0.04$).

In first trimester decidua both proteins were localized in the glandular epithelium. Moderate IKKα immunoreactivity was observed in the decidualized stromal cells and in the endothelium of some blood vessels. NIK was also localized to the decidualized stromal cells although immunoreactivity was faint. NIK was absent from the decidual endothelium. Figures 21 c&d and 22 c&d show NIK and IKKα immunolocalization in first trimester decidua, respectively.

Figure 21: Immunohistochemical localization of NIK in human endometrium and first trimester decidua. Pictures are shown at two magnifications (x20 and x40). **(a and b).** Secretory endometrium. NIK is present in the glandular epithelium and endothelial cells. Little immunoreactivity is present in the stroma. Arrows show location of endothelium. **(c and d).** First trimester decidua. Immunoreactivity is present in the glandular epithelium with faint immunostaining present in decidualized stromal cells. **(e).** Negative control. Primary antibody replaced with rabbit immunoglobulin at equimolar concentration. Scale bars=100μm. Scale bar in (d) also relates to (b); scale bar in (e) relates to (a) and (c).

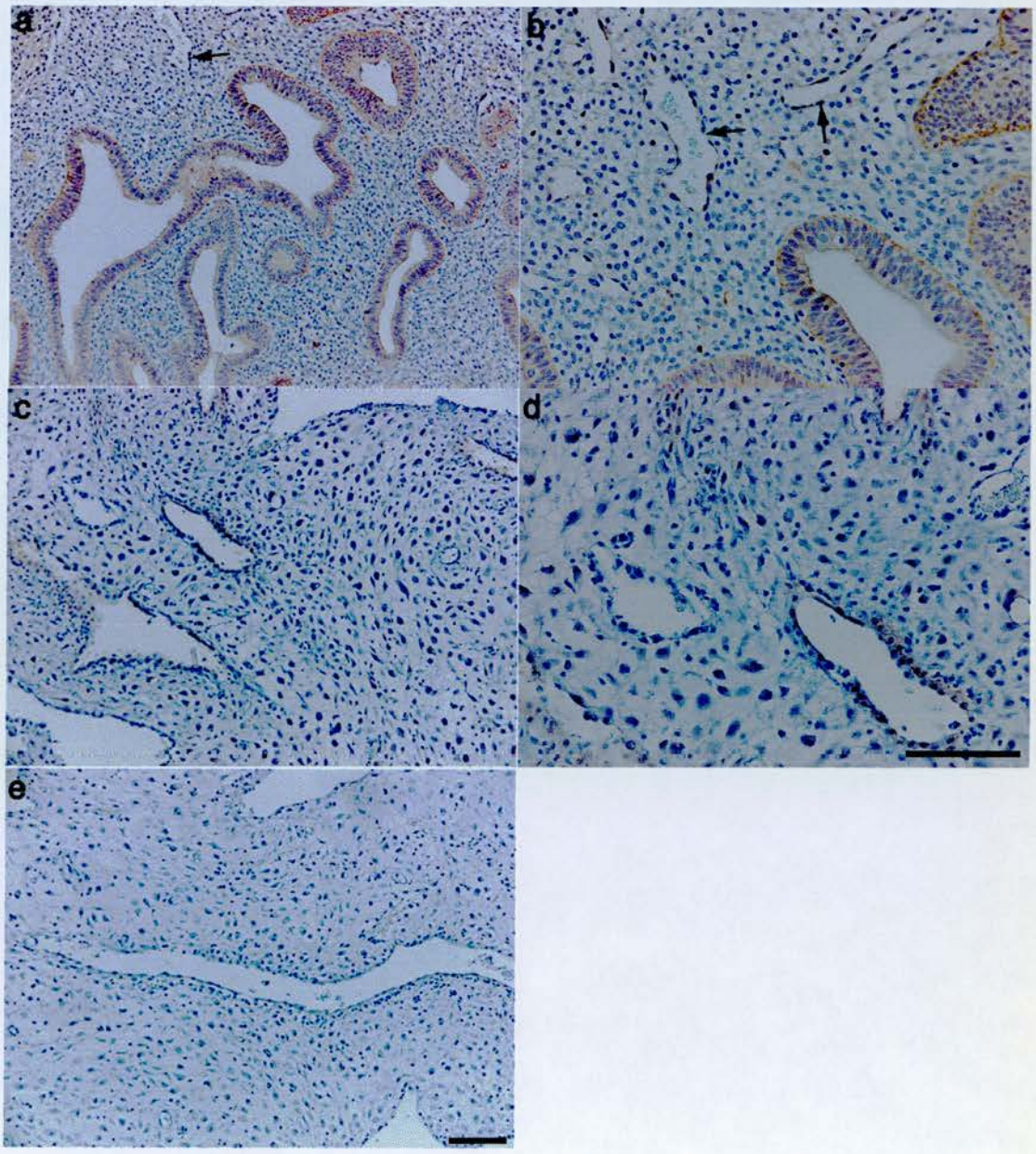
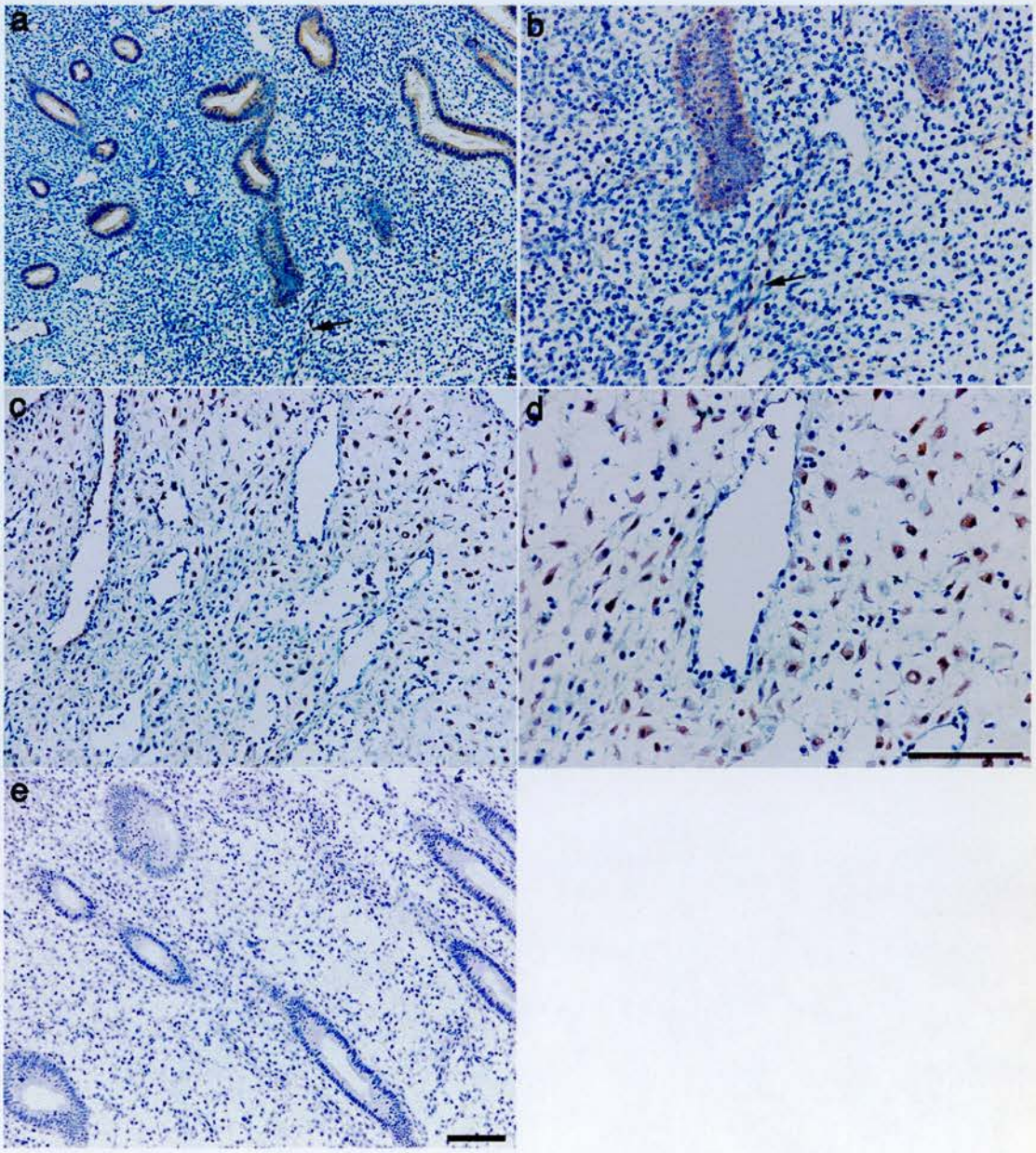

a
b
c
d
e

Figure 22: Immunohistochemical localization of IKKα in human endometrium and first trimester decidua. Pictures are shown at two magnifications (x20 and x40). **(a and b).** Proliferative endometrium. IKKα immunoreactivity is present in the glandular epithelium and endothelial cells. Little immunoreactivity is present in the stroma (only adjacent to luminal epithelium – not shown). Arrows show location of endothelium. **(c and d).** First trimester decidua. Immunoreactivity is localized mainly to the decidualized stromal cells and is also present in the glandular epithelium. Some endothelial immunoreactivity is also present (not shown). **(e).** Negative control. Primary antibody replaced with rabbit immunoglobulin at equimolar concentration. Scale bars=100μm. Scale bar in (d) also relates to (b); scale bar in (e) relates to (a) and (c).


a
b
c
d
e

3.3.2 *In vitro* cell culture studies (endometrial epithelial MFE cells)

3.3.2a The effects of the NFκB inhibitors, SN50 and MG132, on IL-8, COX-2 and IκBα mRNA expression

The data described below represents the mean values from two separate experiments. All treatments were for 24 hours. Treatment of MFE cells with IL-1 caused IL-8 mRNA expression to be raised 3 fold above control values. However, this was not prevented by the addition of the NFκB inhibitors, SN50 and MG132. MG132 alone increased IL-8 mRNA by 140 fold with a further increase of 2.4 fold in the presence of IL-1. SN50 alone had little effect on IL-8 expression but, in the presence of IL-1, mRNA levels were raised 100 fold. (Figure 23).

COX-2 mRNA expression changed in a similar fashion although the effects of the inhibitors were reduced. An increase of 2 fold was observed upon treatment with IL-1 alone. MG132 alone, and with IL-1, caused increases of 17 and 37 fold, respectively. Again, treatment with SN50 alone had little effect. Treatment with IL-1 and SN50 caused an 11 fold increase in COX-2 mRNA expression. (Figure 24).

As expected IL-1 caused an increase in IκBα expression. This was not reduced by treatment with either SN50 or MG132. Neither of these inhibitors affected IκBα mRNA expression. (Figure 25).

Figure 23

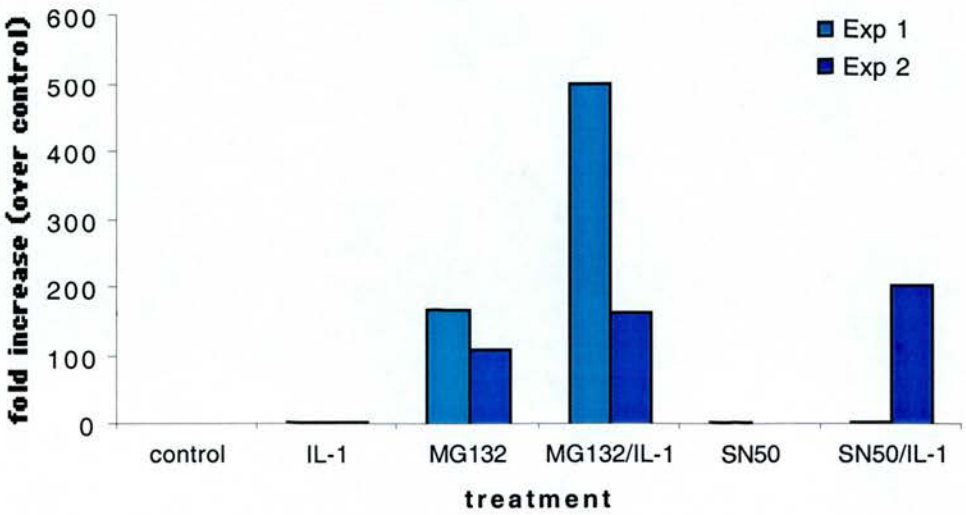
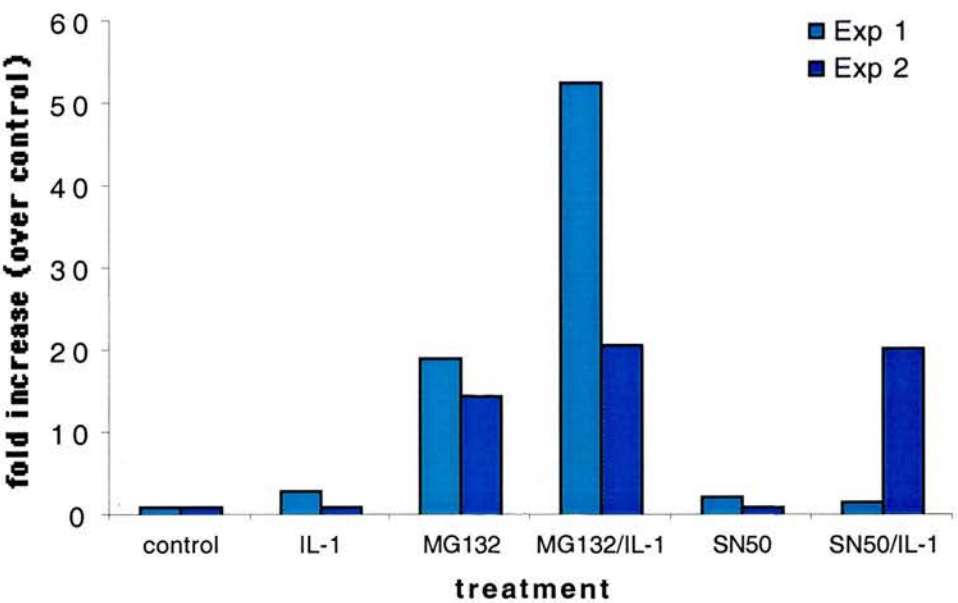


Figure 24



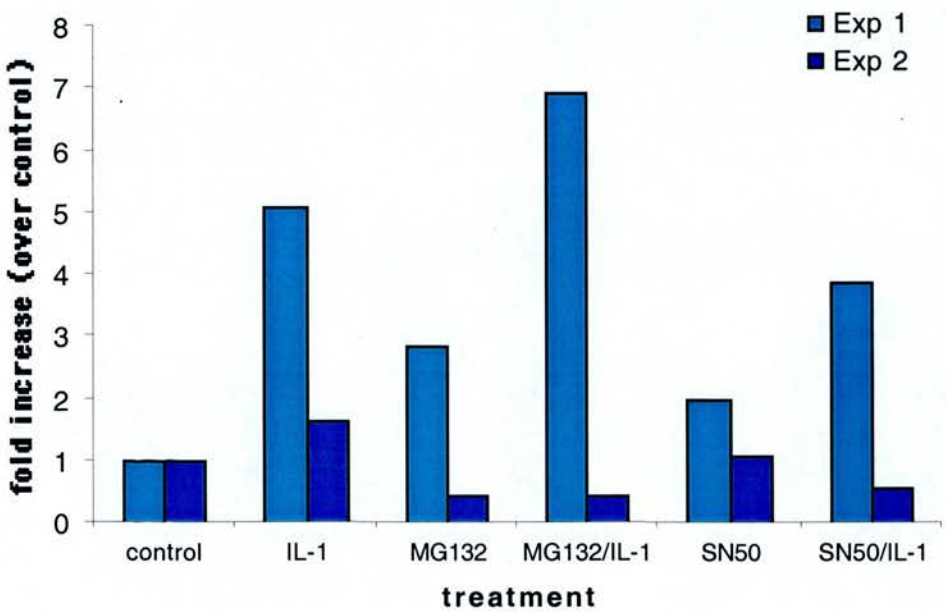


Figure 25: IκBα mRNA expression in MFE cells. The results of two separate experiments are shown (Exp 1 = Experiment 1; Exp 2 = Experiment 2). Data are presented as the fold increase in IκBα mRNA expression relative to control levels. Cells were treated with the NFκB inhibitors, MG132 and SN50, in the absence and presence of interleukin-1 (IL-1). All treatments were for 24 hours. As only two experiments were performed statistical analysis was not possible.

3.3.2b The effects of the NFκB inhibitors, SN50 and MG132, on IL-8 protein expression

The IL-8 protein expression profile (Figure 26) in the presence of IL-1 and the NFκB inhibitors was similar to that of the mRNA. IL-1 increased IL-8 protein expression 2 fold compared to control values. MG132 alone increased protein levels 43 fold with a further increase of 1.7 fold in the presence of IL-1. SN50 alone, and in the presence of IL-1, caused a 3 and 18 fold upregulation of IL-8 protein expression, respectively.

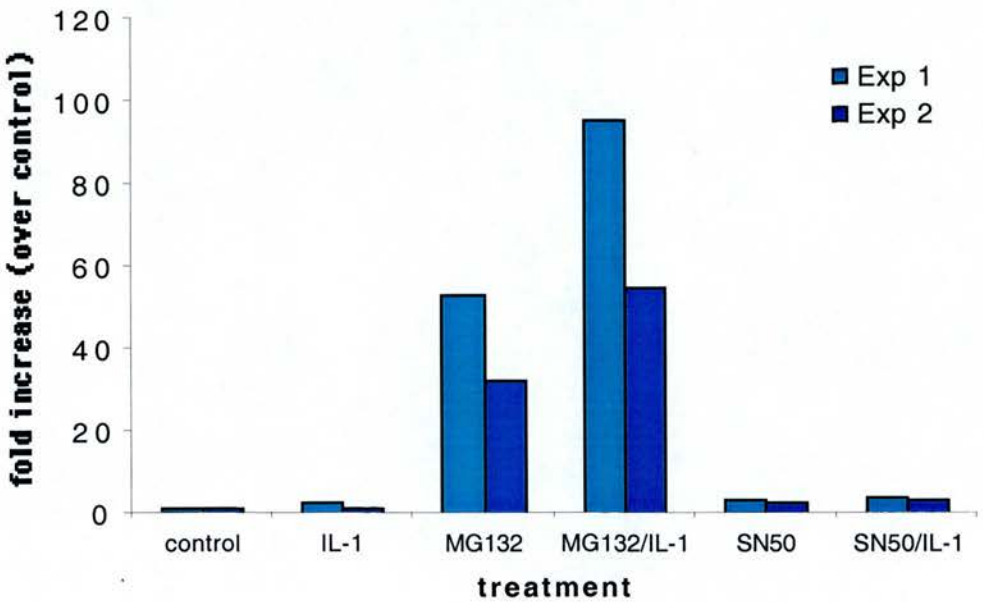


Figure 26: IL-8 protein expression in MFE cells. The results of two separate experiments are shown (Exp 1 = Experiment 1; Exp 2 = Experiment 2). Data are presented as the fold increase in IL-8 protein expression relative to control levels. Cells were treated with the NFκB inhibitors, MG132 and SN50, in the absence and presence of interleukin-1 (IL-1). All treatments were for 24 hours. As only two experiments were performed statistical analysis was not possible.

3.4 Discussion

In endometrium, high progesterone levels are associated with immunosuppressive effects while progesterone withdrawal premenstrually initiates the inflammatory events associated with menstruation. The molecular mechanisms that allow progesterone to mediate immunosuppression and, its withdrawal, to precipitate inflammatory-like events are unclear. The NFκB pathway regulates the expression of several molecules involved in menstruation and implantation and is also inhibited by progesterone. The results presented in this chapter suggest that intermediate molecules in the NFκB activation pathway (see Chapter 1, Figure 4) are differentially regulated during the cycle and the results accord with a modulating role of progesterone.

Menstruation – A role for NFκB?

IκBα is the key molecule involved in the activation of NFκB. The phosphorylation of IκBα by upstream kinases occurs in the presence of an appropriate stimulus. IκBα is then ubiquitinated and degraded by the proteasome allowing free NFκB to enter the nucleus where it regulates gene transcription. Expression of IκBα is upregulated by NFκB activation allowing self-limitation of inflammatory actions (Sun, et al., 1993). Additionally, the steroid hormones, dexamethasone and progesterone, exert some of their anti-inflammatory effects by upregulating IκBα expression (Auphan, et al., 1995; Scheinman, et al., 1995a; Wissink, et al., 1998).

Figure 27 details the role of the NFκB pathway in menstruation. Expression of IκBα mRNA is greater in perimenstrual endometrium than in endometrium from other stages of the cycle. Upregulation of IκBα as a result of high progesterone levels is not possible at this time as progesterone concentrations have declined due to the demise of the corpus luteum (see Figure 13). Instead, it is likely that activation of NFκB during menstruation results in increased IκBα expression. NFκB activation is likely to occur as progesterone concentrations fall premenstrually, relieving the inhibition of the pathway. This would be likely to mediate, at least partly, the

upregulation of inflammatory molecules such as IL-8, MCP-1 and COX-2 which has been reported to occur premenstrually and in a model of progesterone withdrawal (Critchley, et al., 1999; Jones, et al., 1997). Each of these molecules has been reported to be upregulated by NFκB in other systems (Adcock, et al., 1997; Martin, et al., 1997; Mauviel, et al., 1992). NFκB also contributes to the regulation of other molecules which are involved in menstruation e.g. MMPs, IL-1, TNFα (Hiscott, et al., 1993; Shakhov, et al., 1990; Vincenti, et al., 1998). In addition, the increased production of inflammatory mediators during menstruation is likely to further activate NFκB. For example, endothelin-1, PGE_2 and IL-1 are known stimuli of the pathway (Gallois, et al., 1998; Muroi and Suzuki, 1993; Osborn, et al., 1989). As detailed previously, NFκB activation is preceded by IκBα ubiquitination. Ubiquitin immunoreactivity is reported to increase in the endometrial glandular epithelial cells in the late secretory phase (Bebington, et al., 1999). However, this is unlikely to be related to increased ubiquitination of IκBα (and, hence, NFκB activation) as the ubiquitin was detected in the nuclei, rather than the cytoplasm, of the cells.

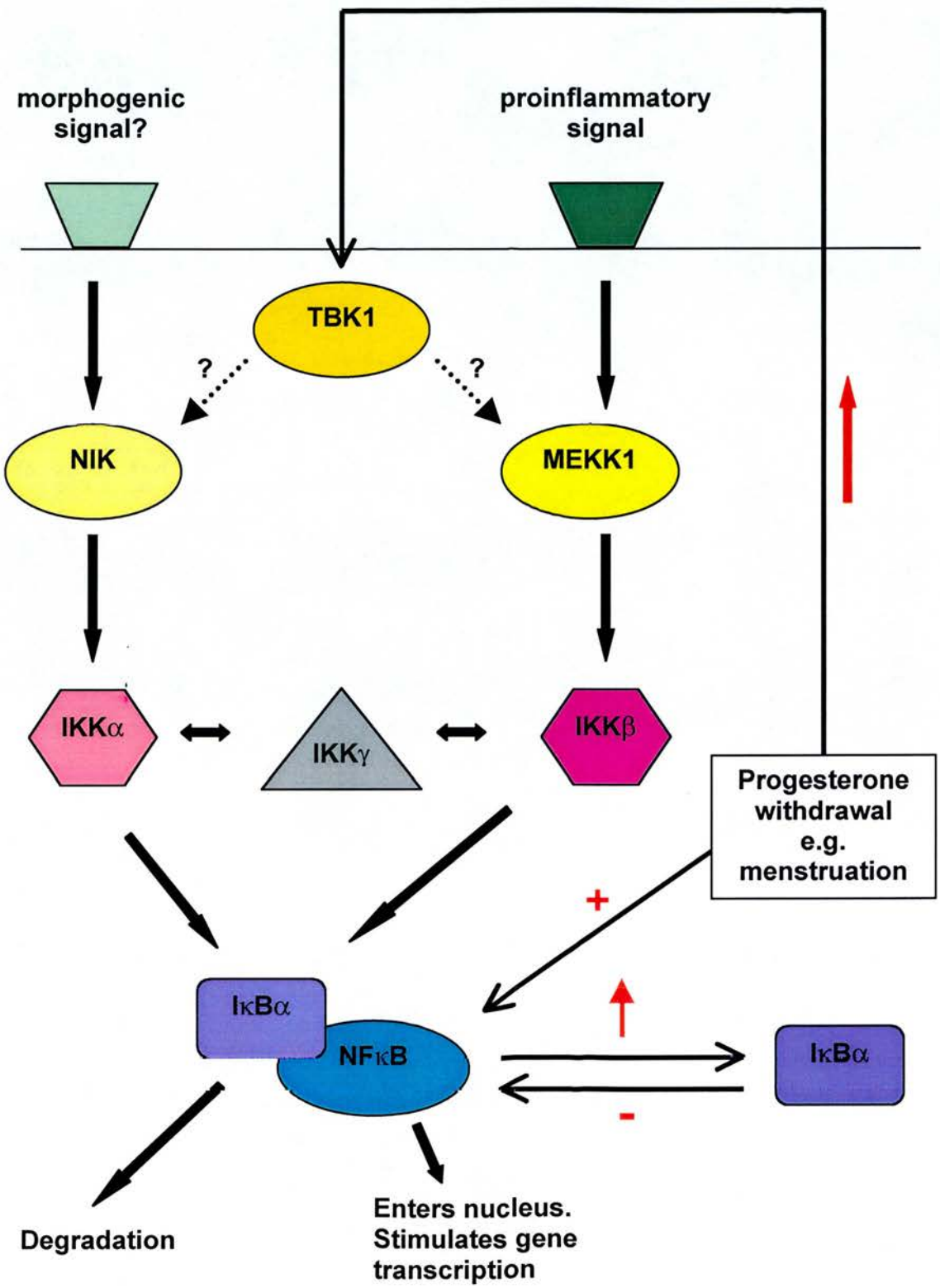
Activation of NFκB during menstruation is also suggested by the observation that mRNA expression of the IKK-like kinase, TBK1, is increased in the perimenstrual phase relative to the secretory phase. This kinase is 48% homologous to an inducible IKK identified in mouse macrophages (Pomerantz and Baltimore, 1999). Its mRNA expression profile in endometrium suggests upregulation as a result of progesterone withdrawal and implies a role in menstruation. Alternatively, the increased production of inflammatory mediators such as IL-1 during menstruation may stimulate TBK1 expression. This would be consistent with induction of the mouse inducible IKK by IL-1 and LPS (Shimada, et al., 1999).

In the secretory phase of the cycle progesterone receptor expression is maintained in the endometrial stromal cells, particularly those in the perivascular region (Wang, et al., 1998). This suggests that the effects of progesterone withdrawal on the NFκB pathway will occur primarily via actions on these cells. The perivascular cells express several chemokines and their close proximity to the blood vessels suggests that they are likely to have a role in the vascular events involved in menstruation. The

immunolocalization of IKKα and NIK in endometrium shows that these elements of the NFκB cascade are not present in the fibroblast cells of the perivascular region (although they are present in endothelium). Immunohistochemical studies focusing on the expression of IKKβ and TBK1 are necessary to determine if these kinases are present in the perivascular region and also, to clarify the effects of progesterone withdrawal on the potential expression of these kinases by the perivascular cells.

When menstruation ceases NFκB activity is likely to be reduced. This might occur as a result of increasing oestradiol concentrations (ER has also been reported to interact with NFκB (McKay and Cidlowski, 1998)) and also, sloughing of the tissue involved in the inflammatory response.

Figure 27: The NFκB pathway in the perimenstrual phase. Prior to menstruation progesterone levels decline rapidly and this is likely to release progesterone-mediated inhibition of the NFκB pathway. The resultant activation of the pathway is likely to contribute to the proinflammatory mechanisms associated with menstruation. In the perimenstrual phase, IκBα mRNA expression is increased suggesting that NFκB is activated at this time. Also, mRNA expression of an IKK-like kinase, TBK1, is increased suggesting a role in menstruation.


morphogenic signal?
proinflammatory signal
TBK1
?
?
NIK
MEKK1
IKKα
IKKγ
IKKβ
Progesterone withdrawal e.g. menstruation
+
IκBα
NFκB
IκBα
-
Degradation
Enters nucleus. Stimulates gene transcription

Pregnancy – Suppression of NFκB activity?

As detailed above progesterone has local immunosuppressive actions during pregnancy. During this time NFκB would be expected to be inhibited to prevent an inappropriate inflammatory response which would be detrimental to the fetus. However, high progesterone levels exert differential effects on the expression of NFκB pathway intermediates in first trimester decidua. This is highlighted in Figures 28 and 29.

The IKK complex is responsible for the phosphorylation of IκBα and consists of two protein kinases, IKKα and IKKβ, and the scaffolding protein, IKKγ. IKKα and IKKβ have been reported to have different functions with IKKα thought to be involved in mediating morphogenic signals to NFκB (Hu, et al., 1999; Takeda, et al., 1999) while IKKβ is primarily involved in proinflammatory signalling (Delhase, et al., 1999; Li, et al., 1999). This suggests that the two kinases may also have divergent roles in endometrium. This is supported by the observation that IKKα mRNA expression is higher in first trimester decidua than in endometrium whereas IKKβ levels are decreased in decidua. NIK and MEKK1, two kinases shown to interact with the IKK complex, are thought to preferentially phosphorylate IKKα and IKKβ, respectively (Ling, et al., 1998; Nakano, et al., 1998). Interestingly, these kinases are differentially regulated in endometrium similar to their target proteins. Expression of NIK mRNA is increased in decidua relative to endometrium while MEKK1 mRNA levels are downregulated. Messenger RNA expression of the scaffolding protein, IKKγ, is significantly lower in decidua relative to secretory phase endometrium. IKKγ is thought to interact primarily with IKKβ (Rothwarf, et al., 1998) and so the decreased levels in decidua may be linked to the reduced expression of IKKβ.

Figure 28: Differential expression of mRNA for NFκB pathway intermediates in first trimester decidua relative to secretory endometrium. Data is presented as the log of decidual mRNA expression:secretory mRNA expression. Both NIK and IKKα are expressed at a greater level in decidua than in endometrium. In contrast, MEKK1 and IKKβ are decreased in decidua. IKKγ mRNA expression is also reduced in decidua while IκBα and TBK1 are increased. 'n' numbers are shown above bars (number of decidua/number of secretory endometrium). Statistical analysis relating to expression of NFκB intermediates throughout the menstrual cycle and in first trimester decidua is presented in Figures 14-20.

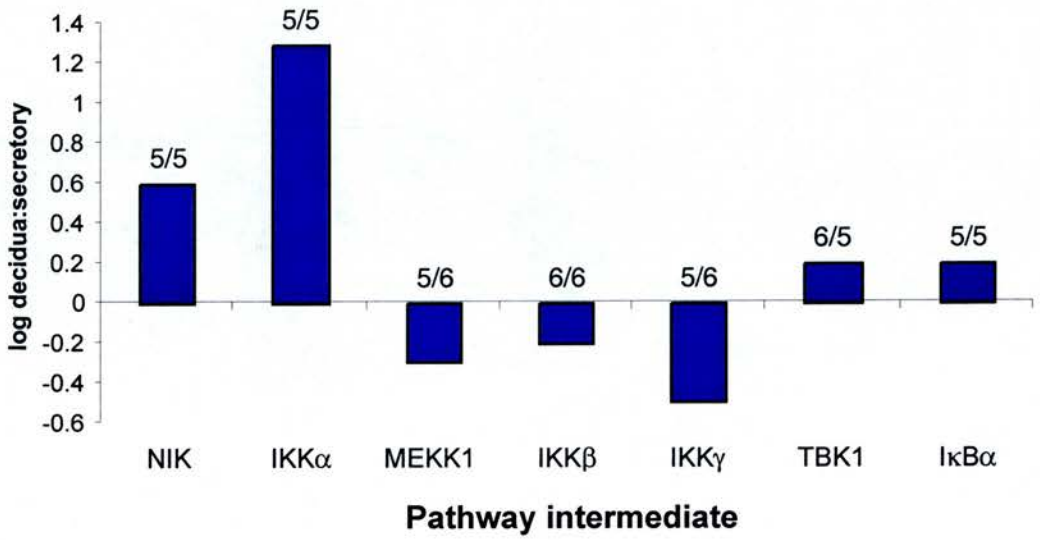
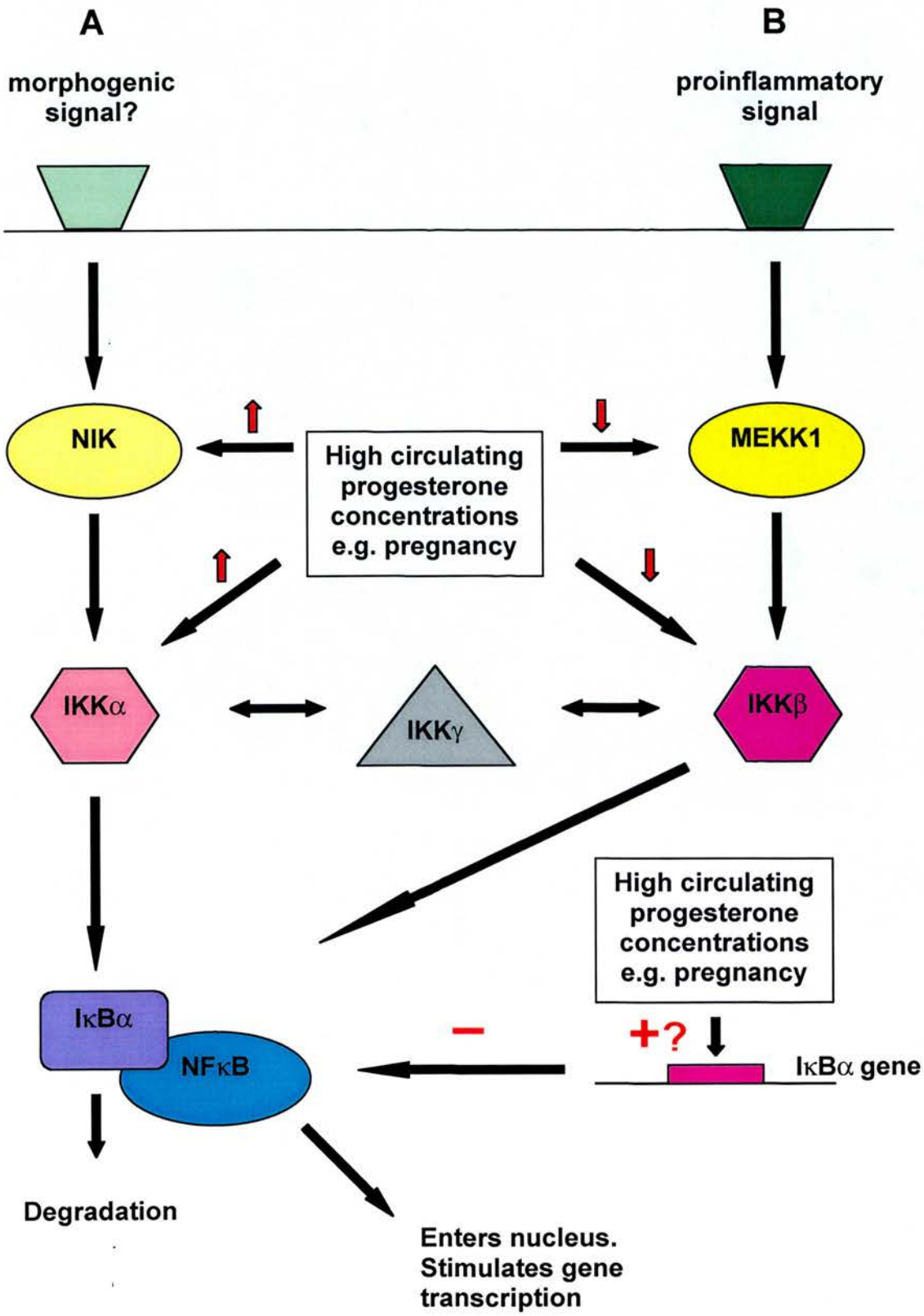

log decidua:secretory
1.4
1.2
1
0.8
0.6
0.4
0.2
0
-0.2
-0.4
-0.6
5/5
5/5
5/6
6/6
5/6
6/5
5/5
NIK
IKKα
MEKK1
IKKβ
IKKγ
TBK1
IκBα
Pathway intermediate

Figure 29: The NFκB pathway in early pregnancy. In the presence of the high progesterone levels of early pregnancy there is differential expression of intermediate molecules in the NFκB pathway. The expression of NIK and IKKα (Pathway A) is upregulated in decidua while the expression of MEKK1 and IKKβ (Pathway B) is reduced. The MEKK1-IKKβ pathway is thought to mediate proinflammatory signals to NFκB and activation of this pathway is likely to be detrimental to the fetus. In contrast, the NIK-IKKα pathway may be involved in decidual growth and differentiation and also, in the expression of molecules vital to successful pregnancy (e.g. COX-2).


A
morphogenic signal?
B
proinflammatory signal
NIK
MEKK1
High circulating progesterone concentrations e.g. pregnancy
IKKα
IKKγ
IKKβ
High circulating progesterone concentrations e.g. pregnancy
IκBα
NFκB
−
+?
IκBα gene
Degradation
Enters nucleus. Stimulates gene transcription

The apparent differential regulation of the signalling pathways to NFκB has several implications. First, as proinflammatory signalling to NFκB is believed to occur via IKKβ it is interesting that this kinase and MEKK1, its activating kinase, are decreased in first trimester decidua (Figure 29, Pathway B). This suggests that the proinflammatory signalling pathway to NFκB is inhibited during pregnancy and is consistent with the local immunosuppressive effects of progesterone. Second, the increased expression of IKKα and NIK (Figure 29, Pathway A) suggests that signalling to NFκB is not universally inhibited by progesterone. Although proinflammatory mechanisms are suppressed during pregnancy, decidua is the source of many molecules which are thought to be beneficial to pregnancy e.g. COX-2, GM-CSF (Jokhi, et al., 1994; Psychoyos, et al., 1995). These can be upregulated by NFκB activation (Adcock, et al., 1997). Also, the NFκB pathway may be involved in the growth and differentiation processes that occur in first trimester decidua with upregulation of NFκB reported to occur during decidualization in the rat (Thienel, et al., 2000).

IKKα and NIK proteins were detected in endometrium and decidua by immunohistochemistry. Both were detected primarily in the glandular epithelium of endometrium. Expression of IKKα and NIK protein in endometrium did not vary during the menstrual cycle and this is consistent with mRNA levels. In decidua both proteins were also present in the decidualized stromal cells. The immunolocalization of IKKα and NIK proteins in the stromal cells of decidua, but not endometrium, is in keeping with the increased mRNA expression of IKKα and NIK in first trimester decidua. The only previous study investigating NFκB activity in endometrium detected the p65 (Rel A) subunit of NFκB in glandular epithelium (and stroma) and suggested that it had a role in the upregulation of LIF and IL-6 during the implantation window (Laird, et al., 2000). The identification of IKKα and NIK in these cells confirms that some upstream molecules in the NFκB signalling pathway are also present. In addition to involvement in LIF and IL-6 expression, NFκB may be involved in the production of several other molecules by the glandular epithelium e.g. TNFα, prostaglandins (Lumsden, et al., 1984; Tabibzadeh, et al., 1995b). IKKα

and NIK are also expressed by the endothelial cells of endometrium. Angiogenesis is involved in menstruation and implantation (Rogers, et al., 1992; Smith, 1998) and NFκB activation has a role in modulating the expression of angiogenic factors by endothelial cells (Yoshida, et al., 1997). Also, NFκB has been reported to upregulate expression of adhesion molecules such as VCAM-1 and so, may have a role in leukocyte infiltration of the endometrium (Iademarco, et al., 1992).

The effects of progesterone on endometrial IκBα expression remain unclear. IκBα mRNA expression is greater in the secretory phase than in the proliferative phase. There is a further increase in expression in decidua. These increases are coincident with the increasing concentrations of progesterone which occur in the secretory phase and the subsequent maintenance of these levels in early pregnancy. This suggests that progesterone may increase IκBα levels in decidua and may be inhibiting NFκB activation by certain stimuli e.g. IKKβ activity. This is consistent with the results to be presented in chapter 6 (which show increased expression of IκBα in the progesterone receptor expressing T47D cell line as a result of progesterone treatment) and also, with previous studies (Miller and Hunt, 1998; Wissink, et al., 1998). Alternatively, the increased expression of IκBα mRNA expression in decidua may occur as a result of NFκB activation via the IKKα-NIK pathway. The mechanisms by which IKKα and IKKβ differentially activate NFκB are currently unexplained. However, one possible explanation is that the kinases may phosphorylate different forms of IκB–NFκB.

This study has focused on the actions of progesterone on the NFκB pathway in the uterus. It should be noted that glucocorticoids are regarded as physiological antagonists of NFκB (McKay and Cidlowski, 1999) and contribute to the regulation of the NFκB pathway in other sites (for mechanisms of regulation see Chapter 1, section 1.3.6). The actions of glucocorticoids on the IKK complex and the upstream kinases have not been fully evaluated and these may differ to the effects of progesterone in the uterus.

NFκB and pathophysiological events in endometrium

In addition to potential roles in endometrial physiology the NFκB pathway is likely to be involved in pathophysiological events. Menstrual problems, such as dysmenorrhea and menorrhagia, and some types of infertility are related to aberrant expression of inflammatory mediators. NFκB may contribute to these problems. In addition, one of the main functions of endometrium is mucosal defence. In this respect it is similar to tissues such as lung and gut. The NFκB system has a role in mucosal defence (as recently reviewed in the case of the intestinal epithelium (Jobin and Sartor, 2000)) and this is likely to be a key function of the pathway in endometrium. The localization of components of the pathway in the epithelium, throughout the cycle, supports the concept that NFκB activation is likely to be involved in the defence against uterine infections.

Production of inflammatory molecules by endometrial epithelial cells – Effects of NFκB inhibition.

Previously, it has been shown that in primary cultures of endometrial epithelial cells the NFκB inhibitors, MG132 and SN50, inhibit IL-1 and TNFα induced IL-6 and LIF production (Laird, et al., 2000). In the MFE endometrial epithelial cell line the expression of IL-8 and COX-2 was increased by IL-1 stimulation. However, simultaneous treatment with MG132 and SN50 did not cause inhibition of this effect. Instead, there was a dramatic increase in IL-8 and COX-2 expression in the presence of MG132. A smaller increase occurred under the influence of SN50 and IL-1. IL-8 protein expression was consistent with the mRNA profile. These results are surprising although there are several possible explanations. First, the proteasome is involved in the degradation of several proteins (Lee and Goldberg, 1998) and inhibition may interfere with pathways other than that of NFκB. This may mean that another pathway involved in the regulation of IL-8 and COX-2 expression has been affected by MG132 treatment. Second, inhibition of NFκB may decrease the production of cytokines that reduce IL-8 and COX-2 expression. This would result in

upregulation of the mediators. Finally, there may be differences between the cytokine control mechanisms present in the MFE cell line and primary cells.

In summary, the results presented in this chapter describe the presence of the NFκB pathway in endometrium. A role for the pathway in menstruation (Figure 27) is suggested by the upregulation of IκBα and the IKK-like kinase, TBK1, in the perimenstrual phase. Additionally, differential regulation of the IκBα kinases, IKKα and IKKβ, and the upstream kinases, NIK and MEKK1, has been observed in first trimester decidua (Figure 29). The proinflammatory signalling pathway, MEKK1-IKKβ, is decreased in decidua suggesting suppression of inflammatory mechanisms at this time. However, upregulation of the NIK-IKKα NFκB activation pathway occurs indicating that signalling can occur via NFκB in decidua. This is likely to have a role in modulating expression of factors, such as COX-2, which are crucial to successful pregnancy.

4: The CD40-CD40 ligand system in human endometrium and first trimester decidua

4.1 Introduction

CD40 and its ligand, CD40L, are involved in proinflammatory signalling via the NFκB signal transduction pathway (Berberich, et al., 1994). Alternatively, the system can activate other signal transduction pathways including JAK-STAT (Hanissian and Geha, 1997). As detailed earlier CD40 is expressed on various cell types while CD40L is expressed predominantly on bone marrow derived cells. Fibroblasts from various sites, including lung and gingiva, have been found to express CD40 (Fries, et al., 1995; Sempowski, et al., 1998). Recent reports have suggested an important role for the CD40-CD40L system in fibroblast physiology. Initially, it was thought that fibroblasts were merely inert, structural cells but it has now been suggested that they interact with cells of the immune system. The CD40-CD40L system may be an important mediator involved in this (Smith, et al., 1997). Some types of fibroblast display increased expression of the proinflammatory cytokines, IL-6 and IL-8, upon engagement of CD40 with CD40L (Sempowski, et al., 1997a; Sempowski, et al., 1997b; Sempowski, et al., 1998). Additionally, increased COX-2 expression and hence, PGE_2 production, results from stimulation of lung fibroblasts via this system (Zhang, et al., 1998).

In a reproductive context there is little information regarding the CD40-CD40L system. Recent work from our laboratory has shown that endometrial, cervical and myometrial fibroblasts display CD40 *in vitro* and that upregulation occurs in the presence of IFNγ. These cells display increased expression of the chemokines, IL-8 and MCP-1, and the cytokine, IL-6, upon stimulation with CD40L (King, et al., 2000b). This is particularly interesting as the role of these molecules in reproductive tissues has been, at least partly, elucidated. As detailed earlier (Chapter 1, section 1.2.2) both IL-8 and MCP-1 are present in the endometrial perivascular area and are involved in leukocyte chemotaxis prior to menstruation (Critchley, et al., 1994; Jones, et al., 1997). IL-8 also has a role in neutrophil chemotaxis during cervical ripening (Kelly, et al., 1992; Sennstrom, et al., 1997). Additionally, IL-6 is expressed in the endometrium from the mid-secretory phase onwards with increased expression as the cycle progresses (Tabibzadeh, et al., 1995a). A role in menstruation has been

suggested and increased production has recently been associated with cervical ripening (Sennstrom, et al., 2000).

The localization of CD40 and CD40L in endometrium, first trimester decidua and myometrium has not previously been established although CD40 has been detected in the squamous epithelium of cervical carcinoma (Altenburg, et al., 1999). The presence of the CD40-CD40L system on fibroblasts *in vivo* in these tissues could provide a link between resident structural cells and infiltrating leukocytes and may be involved in modulation of inflammatory mediator expression. A role in pathophysiological events such as dysfunctional bleeding would also be possible. The results in this chapter detail the protein expression of CD40, and mRNA expression of both CD40 and CD40L, in endometrium and decidua.

4.2 Methods

4.2.1 Human uterine tissue collection

Endometrial (n=37), decidual (n=12) and trophoblast (n=4) biopsies were collected as detailed in 2.1. Non-pregnant myometrium (n=18) was present in full thickness endometrial biopsies (these are described in Chapter 2, section 2.1) and this was utilized in immunohistochemical studies. Human first trimester cervical biopsies (all<63 days gestation) were available from a parallel study supported by the Medical Research Council (grant no: G9620138 and G9406438) addressing the local effects of antiprogestogen and prostaglandin administration. Paraffin tissue sections were available from n=8 'control' cervical biopsies included in the above studies (no antiprogestogen). Biopsies included in this chapter are detailed in Table 9.

Stage of cycle/Tissue biopsy type	Number of biopsies
Menstrual	3
Early proliferative	2
Mid proliferative	8
Late proliferative	5
Early secretory	9
Mid secretory	7
Late secretory	3
Myometrium (non-pregnant)	18
First trimester decidua	12
Trophoblast villi	4
Cervix (pregnant – first trimester)	8

Table 9: Details biopsies used in the studies presented in Chapter 4.

4.2.2 Uterine tissue analyses

4.2.2a RNA extraction and RT-PCR

RNA was extracted from endometrial (n=19), decidual (n=5) and trophoblast (n=4) biopsies and cDNA was prepared. Quantitative PCR was performed in order to determine amounts of CD40 and CD40L mRNA expression. All protocols are detailed in section 2.2.

4.2.2b Immunohistochemistry

CD40 was immunolocalized in endometrial biopsies (n=18) from throughout the menstrual cycle and in first trimester decidua (n=7). CD40 expression was also investigated in cervix (n=8) and myometrium (n=18). Similarly, the cell surface glycoprotein, Thy-1 (expressed by fibroblasts), was localized in endometrium (n=4) and decidua (positive control tissue). CD1a (expressed by dendritic cells) was examined in tonsil (positive control tissue), endometrium (n=4) and cervix (n=3). All immunohistochemistry protocols are detailed in general materials & methods, 2.3.

4.2.2c CD40L Immunohistochemistry

Standard immunohistochemistry protocols (as detailed in section 2.3) were tested on frozen and paraffin embedded tissue sections. Antigen retrieval in paraffin sections was attempted using microwaving, pressure cooking in glycine-EDTA buffer and trypsin digestion. Endometrial biopsies from several cycle stages and decidual biopsies (control and from patients administered with RU486 prior to biopsy collection) were investigated. Tonsil biopsies (positive control) were also examined. The primary antibody was tested at concentrations ranging from 0.5 to 4 μg/ml. In addition, immunolocalization was attempted using a catalyzed amplification technique (Catalyzed Signal Amplification System Peroxidase, Dako Ltd., Cambridge, UK). CD40L transfected cells (Looney cells) were used throughout as a positive control. Cells were seeded onto 8 well chamber slides (poly-L-lysine

coated) and after adherence were treated with brefeldin A (10μg/ml; Sigma, Poole, UK) for 24 hours. This causes the intracellular accumulation of newly synthesized proteins due to inhibition of protein transport from the endoplasmic reticulum to the Golgi apparatus. After incubation with brefeldin A cells were fixed in NBF for 30 minutes at room temperature and then permeablized with Triton X100 at 37°C. The slides of CD40L expressing cells were subsequently treated in an identical manner to tissue sections (although these slides were not included in antigen retrieval steps)

4.2.3 *In vitro* cell culture studies (cell line)

4.2.3a Cell culture

T47D cells were cultured as described previously (see 2.4.2). Table 10 shows the treatments administered. Fetal calf serum stripped of steroid hormones was used throughout.

Treatment	Concentration used	Incubation time
Control	N/A	0, 2, 4, 8 and 24 hours
Progesterone	10^{-6}M	2, 4, 8 and 24 hours

Table 10: Details treatment of the T47D breast epithelial cell line. T47D cells express high levels of progesterone receptor (see Chapter 6).

4.2.3b RNA extraction and RT-PCR

After the above incubations RNA extraction and RT-PCR were performed as detailed earlier (section 2.2). CD40 mRNA expression was measured by PCR.

4.2.4 Statistical analysis

PCR results (tissue only) were analyzed using ANOVA. Individual differences were assigned using Fisher's PLSD test.

4.3 Results

4.3.1 Uterine tissue analyses

4.3.1a CD40 and CD40L mRNA expression in endometrium and decidua

Expression of CD40 mRNA was increased in first trimester decidua in comparison to endometrium from all stages of the menstrual cycle (Figure 30; $P<0.001$). CD40L mRNA expression had a similar expression profile with highest levels in decidua (Figure 31; $P<0.0001$).

Figure 30: CD40 mRNA expression in perimenstrual (peri), proliferative (prol) and secretory (sec) endometrium, decidua (dec) and trophoblast (tb). All sample PCR measurements are related to an internal control (proliferative endometrial sample) and these ratio are presented in the figure (y axis = ratio to calibrator). 'n' numbers are shown above bars. Paired letters indicate statistical significance (a, b, c and d:$P<0.001$).

Figure 31: CD40L mRNA expression in perimenstrual (peri), proliferative (prol) and secretory (sec) endometrium, decidua (dec) and trophoblast (tb). All sample PCR measurements are related to an internal control (proliferative endometrial sample) and these ratio are presented in the figure (y axis = ratio to calibrator). 'n' numbers are shown above bars. Paired letters indicate statistical significance (a, b, c and d:$P<0.0001$).

Figure 30

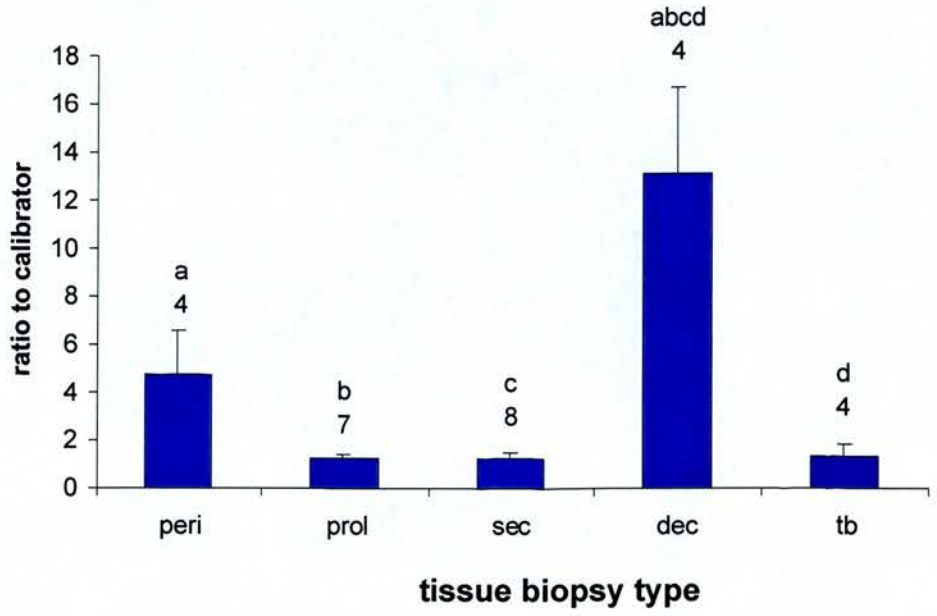
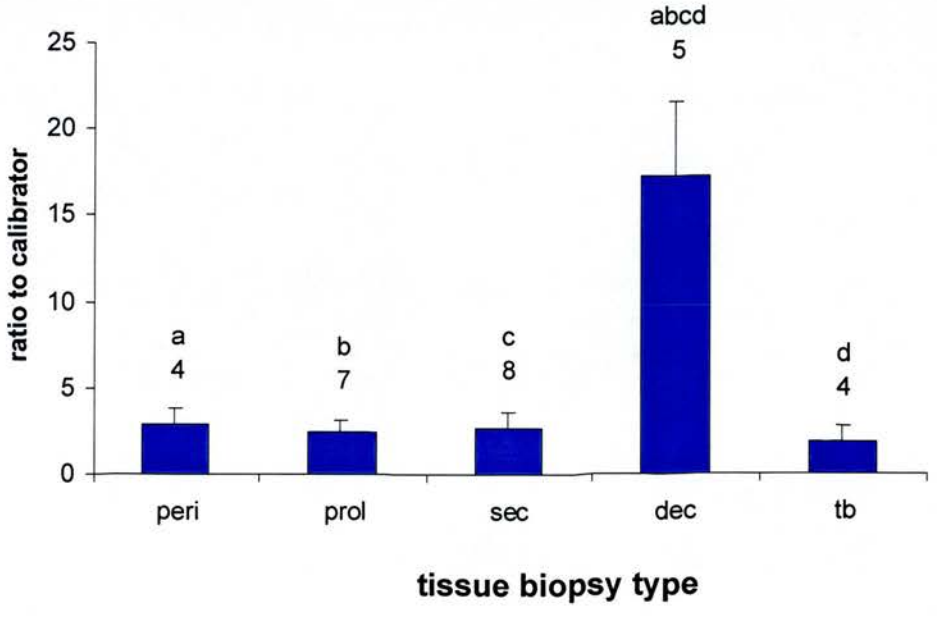


Figure 31



4.3.1b Immunolocalization of CD40 in endometrium, decidua, cervix and myometrium

Positive CD40 immunostaining was detected predominantly in the perivascular region of endometrial biopsies from all stages of the menstrual cycle (Figure 32a&b). CD40 was immunolocalized to this region in both superficial and basal endometrium. The area around the blood vessels that expressed CD40 was several cell layers deep. Moderate immunostaining was also detected on stromal cells, particularly those in the basal and subglandular regions of endometrium (Figure 32b). White blood cell and very faint epithelial immunoreactivity was also observed in some biopsies. No differences were observed in CD40 immunostaining relative to menstrual cycle stage.

In first trimester decidua strong CD40 immunostaining was apparent in the perivascular region. Decidualized stromal cells also expressed CD40. (Figure 32d&e).

CD40 immunoreactivity was present in the perivascular cells of cervix (Figure 33a&b) and myometrium (Figure 33d&e). Faint immunostaining was also observed in fibroblast-like stromal cells. Additionally, CD40 was detected in the basal epithelium of cervical biopsies.

Figure 32: Immunohistochemical localization of CD40 in human endometrium and first trimester decidua. Photomicrographs are at two magnifications. Arrows show the location of blood vessels. **(a).** Early secretory endometrium (serum progesterone: 39.7nmol/L). CD40 immunoreactivity is present in the perivascular area and in some stromal cells. Only very faint immunostaining is found in the glandular epithelium. **(b).** Endometrium. Blood vessels at higher magnification. CD40 stromal immunolocalization is shown (s). **(c).** Endometrium. Negative control. Primary antibody replaced with mouse immunoglobulin at equimolar concentrations. **(d).** First trimester decidua. CD40 is present in the perivascular cells and in the large decidualized stromal cells. **(e).** First trimester decidua. Blood vessels at higher magnification. **(f).** First trimester decidua. Negative control. Scale bars=100μm. Scale bar in (c) also relates to (b); scale bar in (f) relates to (e).

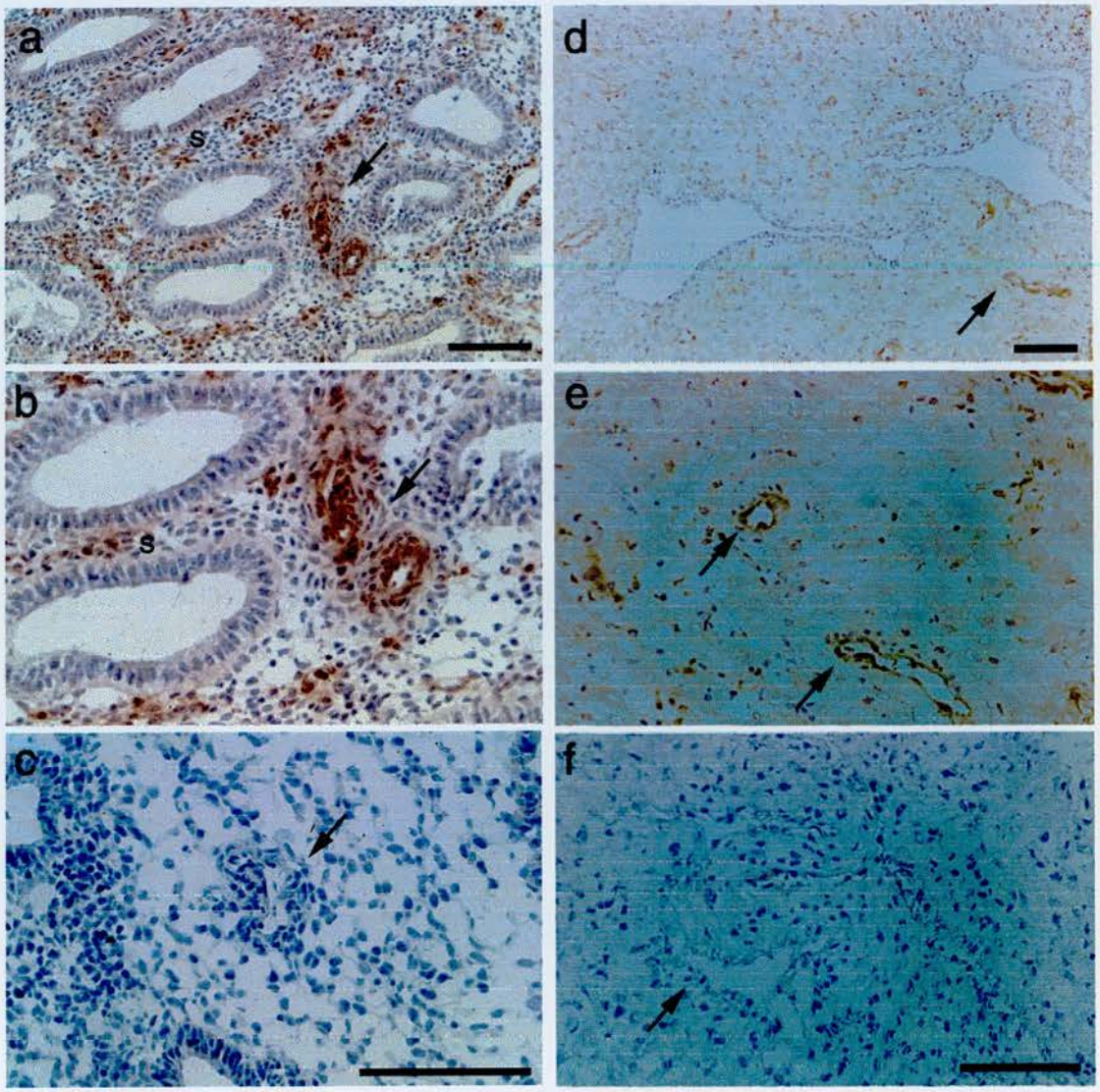
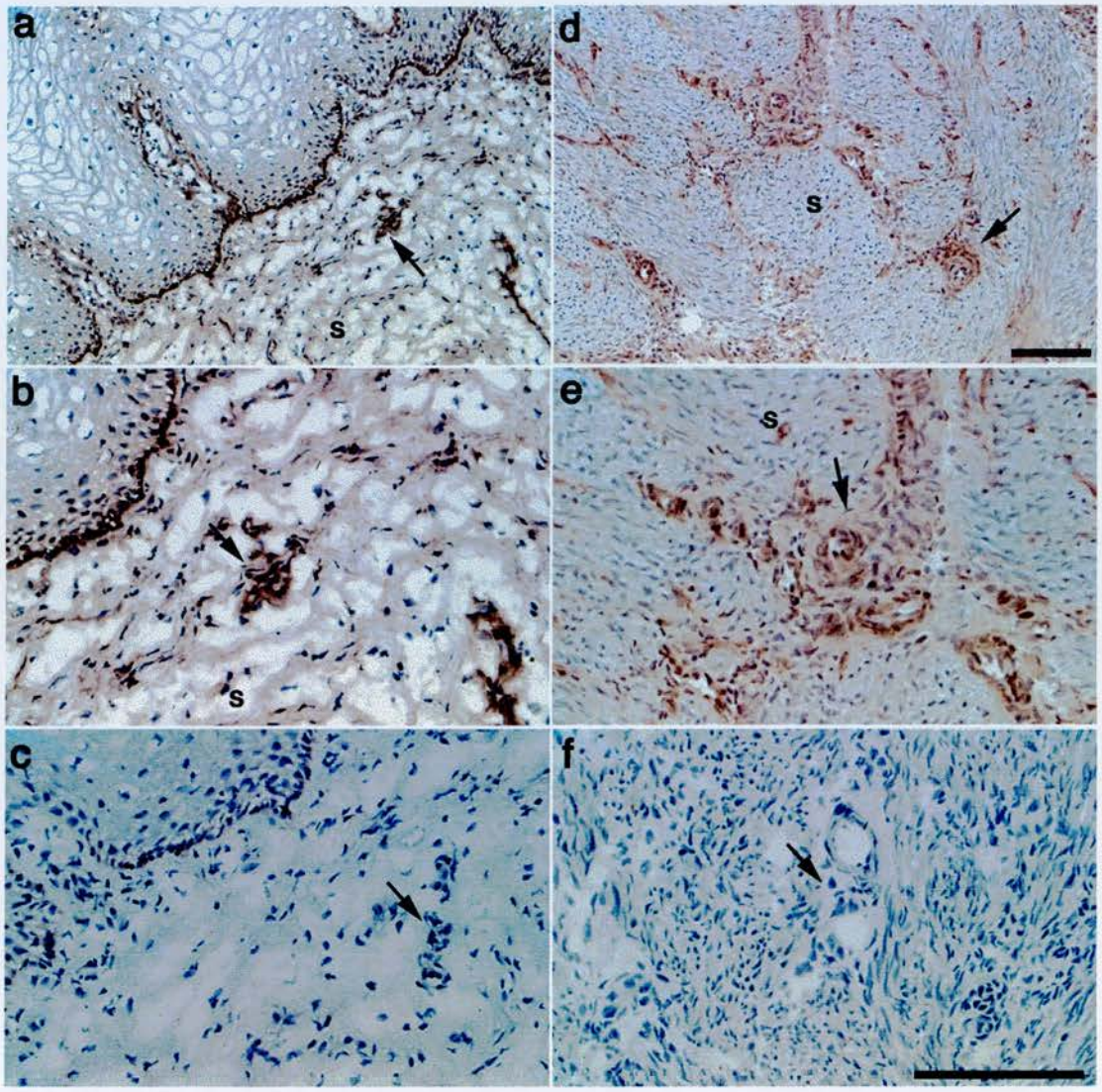

a
s
d
b
s
e
c
f

Figure 33: CD40 immunolocalization in human myometrium (non-pregnant) and cervix (first trimester). Photomicrographs are at two magnifications. Arrows show the location of blood vessels. **(a).** Cervix (day 65 of gestation). CD40 immunoreactivity is present in the perivascular area, some stromal cells (s) and in the basal epithelium. **(b).** Cervix. Blood vessel at higher magnification. **(c).** Cervix. Negative control. Primary antibody replaced with mouse immunoglobulin at equimolar concentrations. **(d).** Myometrium. CD40 is present in perivascular cells and in some fibroblast-like stromal cells (s). **(e).** Myometrium. Blood vessel at higher magnification. **(f).** Myometrium. Negative control. Scale bars = 100μm. Scale bar in (d) also relates to (a); scale bar in (f) relates to (b), (c) and (e).

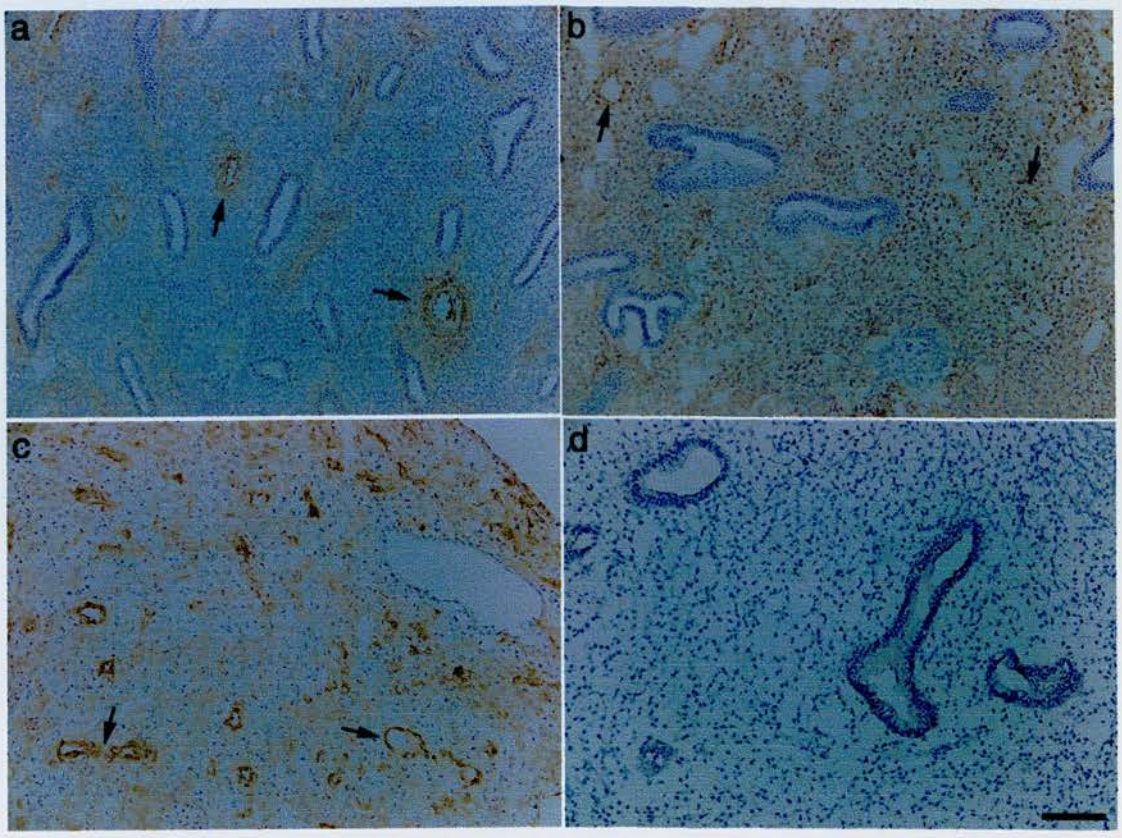

a
s
d
s
b
s
e
s
c
f

4.3.1c Immunolocalization of Thy-1 (expressed by fibroblasts) in endometrium and decidua

A distinctive pattern of Thy-1 stromal immunostaining was observed in all endometrial biopsies studied. Stromal cells in the basalis layer exhibited very little Thy-1 immunostaining (Figure 34a). In contrast, strong immunoreactivity was present in the stromal cells of the functionalis layer (Figure 34b). Positive Thy-1 immunoreactivity was observed in the perivascular area in both functionalis and basalis regions (Figure 34a&b). The perivascular region which expressed Thy-1 was, as for CD40 expression, several cell layers deep.

In first trimester decidua diffuse Thy-1 immunoreactivity was observed in the decidualized stromal cells and particularly, in the perivascular cells (Figure 34c).

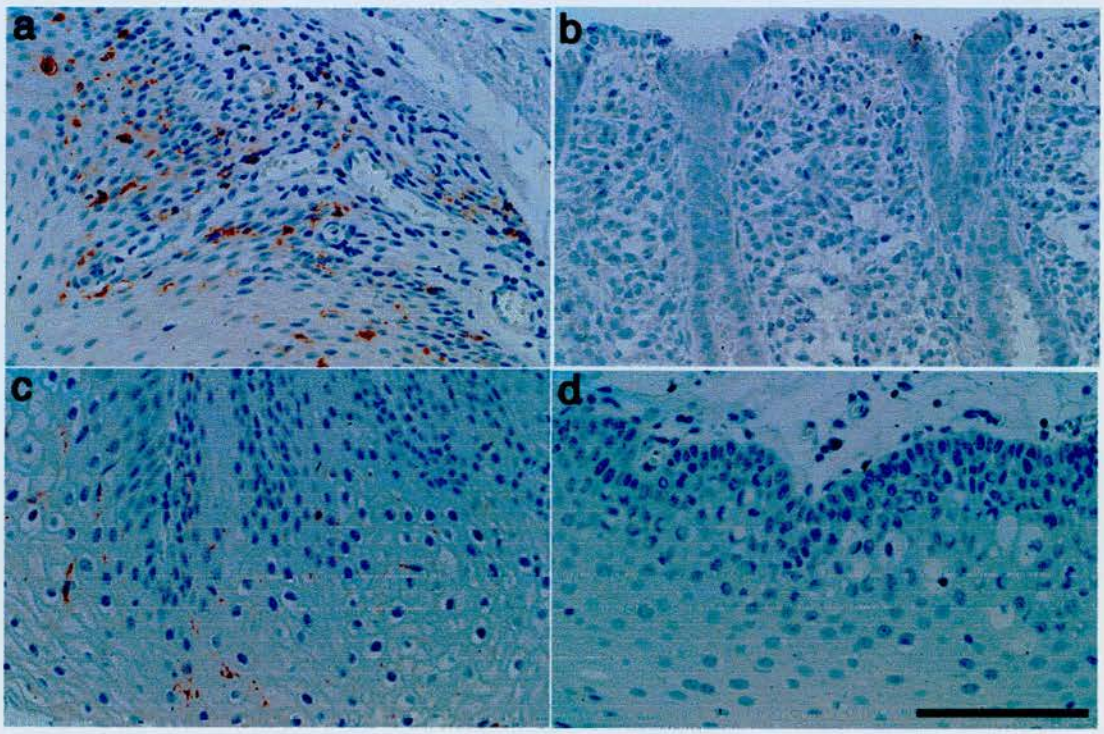
Figure 34: Thy-1 immunolocalization in human endometrium and first trimester decidua. Arrows show the location of blood vessels. **(a).** Basalis endometrium. Thy-1 is present in the cells surrounding the blood vessels and the glandular basement membrane but is not present in the stromal cells. **(b).** Functionalis endometrium. Thy-1 is present in perivascular cells and throughout the stroma. **(c).** First trimester decidua. Immunoreactivity is present in the cells around the blood vessels and in the large decidualized stromal cells. **(d).** Negative control. Primary antibody substituted with mouse immunoglobulin at a concentration of 1μg/ml. Scale bar = 100μm.


a
b
c
d

4.3.1d Immunolocalization of the dendritic cell marker, CD1a, in endometrium

Positive CD1a immunostaining was observed in human tonsil (positive control tissue) in cells infiltrating the epithelium (Figure 35a). Similarly, a small number of cells infiltrating the squamous cervical epithelium displayed CD1a immunoreactivity (Figure 35c). No obvious CD1a expression was observed in endometrium (Figure 35b).

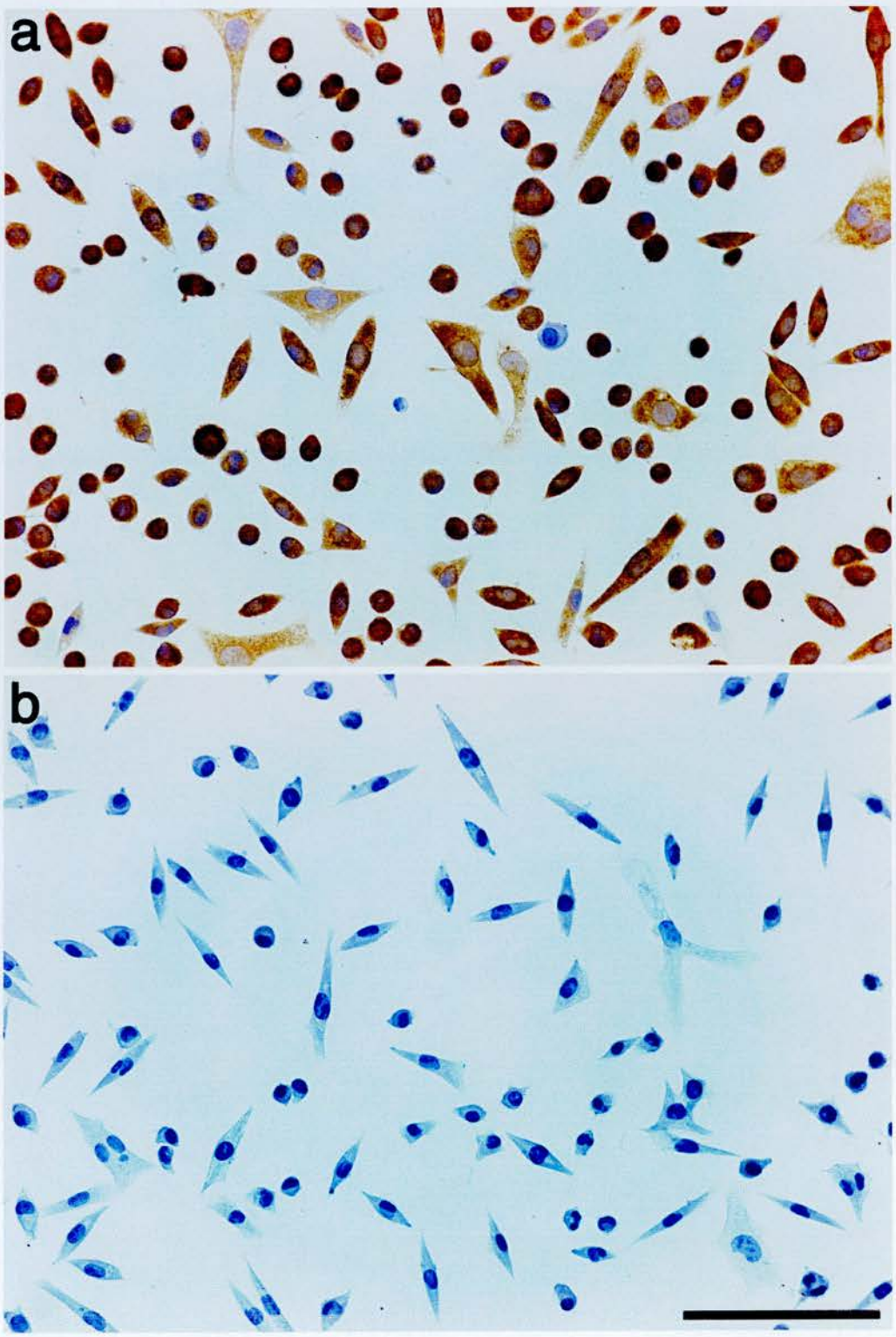
Figure 35: Immunohistochemical localization of the dendritic cell marker, CD1a, in human endometrium, cervix and tonsil. **(a).** Tonsil (positive control). CD1a is present on dendritic cells infiltrating the epithelium. **(b).** Endometrium. No immunostaining was observed. **(c).** Cervix. Immunoreactivity is present on cells infiltrating the squamous epithelium. **(d).** Negative control. Primary antibody substituted with mouse immunoglobulin at a concentration of 2μg/ml. Scale bar = 100μm.


a
b
c
d

4.3.1e CD40L Immunohistochemistry

It was not possible to detect CD40L in endometrium using any of the protocols tested. However, CD40L transfected cells displayed positive immunoreactivity throughout (Figure 36a). This inability to detect CD40L may be due to the transient nature of CD40L expression. Alternatively, although immunolocalization was attempted in biopsies from throughout the menstrual cycle, CD40L may have a very limited expression window in endometrium.

Figure 36: CD40L immunolocalization in CD40L-transfected cells. **(a).** CD40L is present in the cytoplasm of the cells. Although CD40L is normally expressed on the cell membrane, treatment with brefeldin A ensures that the cells retain CD40L in the cytoplasm (see 4.2.2c, immunohistochemistry). **(b).** Negative control. The primary antibody is substituted with an equimolar concentration of mouse immunoglobulin. Scale bar=100μm.


a
b

4.3.2 *In vitro* cell culture studies (breast epithelial T47D cell line)

4.3.2a Effects of progesterone on CD40 mRNA expression in T47D cells

Progesterone had no effect on CD40 mRNA expression by T47D cells during the 0-24 hour timecourse (Figure 37).

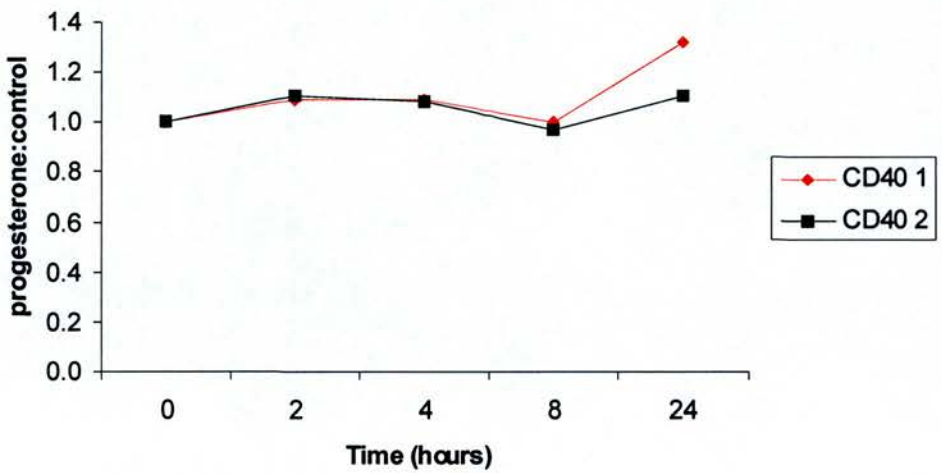


Figure 37: CD40 mRNA expression in T47D cells. Cells were treated with and without progesterone for 0-24 hours (x-axis). Data is shown as the ratio of progesterone:control values (y-axis) and the results of two separate experiments are shown. Progesterone had no effect on the expression of CD40 mRNA in T47D cells.

4.4 Discussion

The results in this chapter describe the presence of CD40 on the perivascular cells of endometrium. This finding suggests a role for the CD40-CD40L system in the regulation of the inflammatory events which are associated with menstruation and implantation (Finn, 1986; Kelly, 1994). Additionally, CD40 is present in the perivascular region of cervix and myometrium and may have a role in cervical ripening and parturition, which are also proinflammatory events.

CD40 has been detected *in vitro* on fibroblasts from several sites including endometrium, myometrium and cervix (King, et al., 2000b). The synthesis of the chemokines, IL-8 and MCP-1, and the cytokine, IL-6, by these fibroblasts is increased upon CD40 engagement. In endometrium, MCP-1 and IL-8 expression is upregulated in the perivascular area premenstrually (Jones, et al., 1997). Increased IL-8 mRNA and protein expression is also observed in an *in vivo* model of progesterone withdrawal (Critchley, et al., 1999). The identification of CD40 on the perivascular cells suggests that activation of this system may be responsible for the upregulation of these chemokines prior to menstruation. Additionally, COX-2 increases perivascularly in the late secretory phase of the menstrual cycle and after progesterone withdrawal (Critchley, et al., 1999; Jones, et al., 1997) while perivascular PGE_2 is upregulated in decidua after administration of a progesterone antagonist (Cheng, et al., 1993a). CD40 activation could also be involved in this as increased COX-2 and PGE_2 expression occurs upon engagement of CD40 on lung fibroblasts (Zhang, et al., 1998).

The perivascular region includes endothelial cells, fibroblasts and smooth muscle cells. The nature of the cell types expressing CD40 in this area is unclear although all of the above have been reported to express CD40 (Fries, et al., 1995; Hollenbaugh, et al., 1995; Mach, et al., 1997). However, a recent report (Oliver, et al., 1999) has suggested that decidual perivascular cells (predecidual cells) have ultrastructural characteristics similar to myofibroblasts and has shown immunohistochemically that these cells express both Thy-1 (a molecule expressed by fibroblasts and smooth

muscle cells (Dalchau, et al., 1989)) and a marker of smooth muscle differentiation, α-smooth muscle actin (Darby, et al., 1990). Similar to CD40 expression, Thy-1 is present on several cell layers around the blood vessels and this suggests that the same cells express both CD40 and Thy-1. However, colocalization studies are necessary to confirm that CD40 is expressed on myofibroblasts in the endometrial perivascular region and also, that these are the cells that express IL-8, MCP-1 and COX-2. In first trimester decidua, CD40 is expressed in the perivascular region and there is also more diffuse immunoreactivity present in the decidualized stromal cells. These cells are believed to form from the proliferation and differentiation of the endometrial stromal cells and this, decidual change, begins around the spiral arterioles (Loke and King, 1995). As discussed above, the perivascular cells have a phenotype consistent with that of the myofibroblast and this is also true of the decidualized stromal cell (Oliver, et al., 1999). The expression of both CD40 and Thy-1 by the perivascular cells of the endometrium and the decidualized stromal cells is consistent with this report and suggests that both molecules are expressed by myofibroblasts.

In addition to Thy-1 the expression of the dendritic cell marker, CD1a, was investigated. A previous study has suggested that dendritic cells constitute only a very minor cell population in endometrium with dendritic-like cells identified in an epithelial location in only five of thirty endometrial samples (Kamat and Isaacson, 1987). This finding along with the absence of CD1a immunoreactivity in the few endometrial samples investigated in this study suggests that dendritic cells are unlikely to be a major source of CD40 in endometrium. CD1a expression was observed on cells infiltrating the cervical squamous epithelium and this confirms previous reported data (Poppe, et al., 1998).

In vitro fibroblast CD40 expression is upregulated by IFNγ. In endometrium, expression of CD40 is also likely to be maintained by the presence of an inflammatory mediator. T cells associated with endometrial lymphoid aggregates, present between the bases of glands in the basalis region, have been suggested as a likely source of IFNγ (Tabibzadeh, 1994). If these cells do produce IFNγ they may be responsible for the CD40 stromal staining found in the basalis and subglandular

regions of endometrium (there is less stromal staining in the functionalis). However, the reports suggesting that lymphoid aggregates produce IFNγ have been disputed in a study which states that neutrophils are the main source of endometrial IFNγ (Yeaman, et al., 1998). Neutrophils are closely associated with menstruation (Poropatich, et al., 1987) and may have a role in regulating CD40 expression via IFNγ release. However, this would be in contrast to the finding that CD40 expression is menstrual cycle independent.

The activation of CD40 relies on the presence of CD40L expressing cells at the local site. Although it was not possible to detect CD40L immunohistochemically in this study, there are several cell types capable of expressing CD40L present in endometrium. Resident T cells, macrophages and mast cells are present throughout the menstrual cycle while eosinophils infiltrate the endometrium premenstrually. Activated platelets also express CD40L (Henn, et al., 1998) and these may be present in the uterus during bleeding or infection. Additionally, uterine NK cells are present in the mid-late secretory phase (Jeziorska, et al., 1995; Loke and King, 1995; Poropatich, et al., 1987). In first trimester decidua, uterine NK cells and macrophages are present in greatest number (Loke and King, 1995). It is likely that the function of the CD40-CD40L system in endometrium and decidua involves mediating an interaction between resident structural cells and infiltrating leukocytes.

There are several potential roles for the CD40 system in endometrium. As detailed above, CD40 activation may be involved in the upregulation of chemokines by the perivascular cells in the late secretory phase of the menstrual cycle. This suggests that CD40 may have a role in menstruation by modulating the systems involved in leukocyte recruitment. As the activation of CD40 is likely to be dependent upon the presence of leukocytes it is possible that the system may not be involved in the initial upregulation of chemokine synthesis. Instead, it may be that the system is part of a positive feedback loop which, as leukocytes infiltrate the tissue, allows further chemokine production. This is consistent with the observation that CD40 immunoreactivity is not upregulated in the late secretory phase of the menstrual cycle (i.e. upon progesterone withdrawal). This was further substantiated by the findings

that CD40 mRNA expression did not significantly differ during the menstrual cycle and also, that progesterone had no effect on CD40 mRNA expression in T47D cells. This lack of CD40 regulation by progesterone and its subsequent withdrawal suggests that the pathway is primarily controlled by indirect steroid regulation of the migration of CD40L-expressing leukocytes into endometrium. It is also likely that CD40 is involved in the tissue regeneration that occurs during and after menstruation. Endometrial leukocytes have been suggested to contribute to this via cytokine release and phagocytosis of debris (Salamonsen, 2000). It is also possible that leukocytes expressing CD40L would activate CD40-expressing myofibroblasts in endometrium. Myofibroblasts have contractile activity and are thought to be involved in wound healing (Schurch, et al., 1992). The actions of the CD40-CD40L system during menstruation are likely to be mediated, at least in part, by activation of the NFκB pathway. As described in chapter 3 (section 3.4), NFκB activation in the perivascular region, as a result of progesterone withdrawal, is most likely to be mediated via the proinflammatory kinase, IKKβ, or the IKK-like kinase, TBK1 .

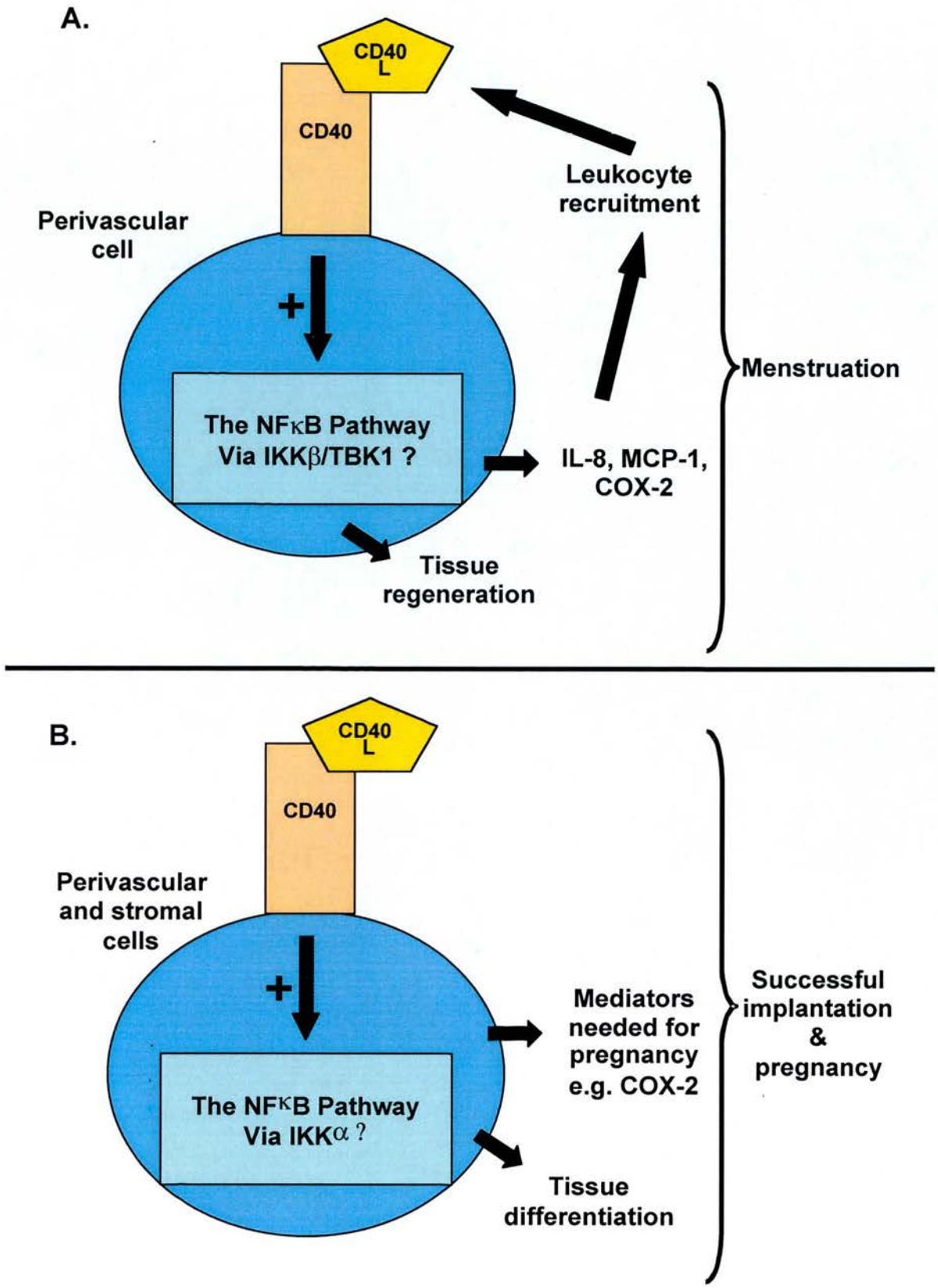
In first trimester decidua, CD40 is present in higher levels than in endometrium. Also, the mRNA expression profile of CD40L suggests that it is present at greatest levels in first trimester decidua (mRNA levels are very low in endometrium). The presence of an apparently proinflammatory system in decidua is surprising. However, the striking similarity between the patterns of CD40 and CD40L mRNA expression and those of NIK and IKKα is interesting (see chapter 3, section 3.3, Figures 15&16). This observation suggests that if the CD40 system activates the NFκB pathway in decidua it may do this via IKKα rather than the proinflammatory IKKβ. As discussed in chapter 3 the consequences of such differential signalling is unclear but it may have a role in the growth and differentiation of decidua and the regulation of molecules such as COX-2 which are vital for successful pregnancy. It should also be noted that the CD40 system can activate signal transduction pathways other than that of NFκB and that this may be involved in endometrial and decidual physiology. The potential roles of the CD40-CD40L system in endometrium and first trimester decidua are summarized in Figure 38. Possible interactions with the NFκB pathway are also detailed.

As described above, CD40 is also present in cervix (pregnant) and myometrium (non-pregnant) and is expressed in the perivascular cells of these tissues. This suggests a role in the proinflammatory events associated with cervical ripening and parturition. The CD40-CD40L system is likely to be involved in the increased expression of IL-8 in the ripening cervix and in the lower segment myometrium during labour (Kelly, et al., 1992; Sennstrom, et al., 1997; Thomson, et al., 1999). In the cervix increased IL-8 production, in synergy with PGE_2, results in the recruitment and activation of neutrophils which is crucial to the ripening process (Colditz, 1990; Rampart, et al., 1989). Cells capable of expressing CD40L are present in both cervix and myometrium. Mast cells and eosinophils are present in the ripening cervix and macrophages are present from late pregnancy onwards (Knudsen, et al., 1997). In myometrium, T cells and macrophages are present at the onset of labour. Mast cells are also present but at lower levels than in the non-pregnant state (Thomson, et al., 1999).

In addition to a role in physiological reproductive events the CD40-CD40L system may be involved in pathophysiological problems. As described in chapter 3, dysfunctional menstrual bleeding is likely to involve the aberrant expression of uterine molecules. For example, there is reduced expression of α-smooth muscle actin in the perivascular region of straight arteries in women suffering from menorrhagia (Abberton, et al., 1999). Inflammatory mediators associated with the perivascular region of endometrium and myometrium (e.g. prostaglandins) may also be associated with menorrhagia and it seems likely that dysregulation of the CD40 system could be involved. Premature labour is associated with increased expression of proinflammatory cytokines in the uterine cavity particularly during infection (Romero, et al., 1990; Romero, et al., 1991). The CD40-CD40L system may be activated during uterine infection and may contribute to the upregulation of cytokines such as IL-6. Recently, CD40 has been detected in cervical carcinoma with CD40L present on infiltrating T cells. CD40-CD40L interactions were suggested as a possible regulator of chemokine expression in carcinoma cells indicating that the CD40 system may be involved in reproductive cancers (Altenburg, et al., 1999).

In summary, this study has identified CD40 on the perivascular cells of the endometrium and other reproductive tissues. The CD40-CD40L system is likely to be involved in proinflammatory events in these tissues (e.g. menstruation, parturition) and in particular, the system is likely to allow interactions between resident structural cells and infiltrating immune cells (Figure 38a). Regulation of inflammatory molecule expression by CD40 is likely to occur via the NFκB pathway. Additionally, the presence of the system in first trimester decidua (Figure 38b) suggests that it may be involved in the regulation of expression of molecules, such as COX-2, which are vital for successful pregnancy.

Figure 38: The role of the CD40-CD40L system in endometrium and first trimester decidua and potential interactions with the NFκB pathway. **(A).** In endometrium, CD40 is expressed on the perivascular cells and is likely to be involved in upregulation of proinflammatory molecule expression by these cells prior to, and during, menstruation. This will encourage the migration of leukocytes to the tissue. These leukocytes are a potential source of CD40L in endometrium. CD40 may be activated by CD40L-expressing leukocytes and this will result in NFκB activation, possibly via IKKβ or TBK1. **(B).** CD40 is likely to be involved in activation of NFκB in decidua via the NIK-IKKα pathway. This may contribute to the expression of inflammatory mediators beneficial to pregnancy. Also, there may be involvement in the growth and differentiation of decidua. It is unclear which cell types express CD40L in decidua although, as with endometrium, leukocytes are a potential source.


A.
CD40 L
CD40
Perivascular cell
Leukocyte recruitment
The NFκB Pathway Via IKKβ/TBK1 ?
IL-8, MCP-1, COX-2
Tissue regeneration
Menstruation
B.
CD40 L
CD40
Perivascular and stromal cells
The NFκB Pathway Via IKKα ?
Mediators needed for pregnancy e.g. COX-2
Tissue differentiation
Successful implantation & pregnancy

5: Secretory leukocyte protease inhibitor (SLPI) expression in human endometrium and first trimester decidua

5.1 Introduction

Secretory leukocyte protease inhibitor (SLPI), a critical neutrophil elastase inhibitor (Thompson and Ohlsson, 1986), is found associated with mucosal surfaces (Franken, et al., 1989) and is reported to have antibacterial, antiviral and antifungal effects (Tomee, et al., 1998). Additionally, it has more generalized anti-inflammatory actions suppressing LPS activation of mouse macrophages (Jin, et al., 1997) and inhibiting MMP production by human monocytes (Zhang, et al., 1997). SLPI also inhibits activation of NFκB and, via this action, is likely to suppress the inflammatory response (Jin, et al., 1997; Lentsch, et al., 1999). SLPI is thus a pleiotropic molecule protecting against inflammatory insult and infection of mucosal surfaces in several ways.

In a reproductive context, SLPI has been detected in human cervical mucosa (Casslen, et al., 1981), term decidua (Denison, et al., 1999b) and seminal plasma (Franken, et al., 1989). This suggests that SLPI contributes to the mucosal defence mechanisms present in the female reproductive tract. SLPI has also been localized to the glandular and luminal epithelial cells of porcine maternal endometrium (Reed, et al., 1996). Additionally, the mRNA has been detected in equine and bovine endometrium during pregnancy (Badinga, et al., 1994). It has been suggested that SLPI acts to maintain the uterine-placental border in these species. Expression was thought to be related to epitheliochorial placentation as SLPI was not detected in the endometrium of mammals with haemochorial placentation (e.g. rat) (Badinga, et al., 1994) although this finding is in contrast to the reported presence of SLPI in human term decidua. Mitogenic effects of SLPI on porcine endometrial epithelial cells have also been reported suggesting that SLPI may act as an autocrine growth promoter (Badinga, et al., 1999).

Previous studies have failed to detect SLPI in non-pregnant human endometrium (Casslen, et al., 1981; Franken, et al., 1989) although SLPI is present in uterine fluid (Casslen, et al., 1981). Successful human implantation and pregnancy demands that there is control of inflammatory responses in the uterus. It seems likely that SLPI

may contribute to this via its antibacterial and anti-inflammatory actions. The results presented in this chapter describe the production of SLPI by endometrium and first trimester decidua. Regulation of SLPI production by cervical and endometrial cell lines is also detailed.

5.2 Methods

5.2.1 Human uterine tissue collection

Endometrial (n=56), decidual (n=24) and trophoblast (n=13) samples were collected as described in 2.1. In addition, endometrium was collected from women using a levonorgestrel intrauterine system (LNG-IUS; n=4) for contraception and heavy menses. The LNG-IUS creates very high local concentrations of progestogen in endometrium (Pekonen, et al., 1992) resulting in decidualization (Critchley, et al., 1998a; Critchley, et al., 1998b). The biopsies utilized in this chapter are detailed in Table 11.

Stage of Cycle/Tissue biopsy type	Number of biopsies
Menstrual	5
Early proliferative	4
Mid proliferative	12
Late proliferative	9
Early secretory	11
Mid secretory	7
Late secretory	8
First trimester decidua	24
Trophoblast villi	13
LNG-IUS	4

Table 11: Details biopsies used in the studies presented in Chapter 5.

5.2.2 Uterine tissue analyses

5.2.2a Tissue culture

Endometrium (n=38), endometrium from LNG-IUS users (n=4), decidua (n=18) and trophoblast (n=13) were cultured as described in 2.4.1. The endometrial and decidual biopsies were cultured in the presence of oestradiol (10^{-8}M); otherwise there were no additional treatments. Culture medium was stored for subsequent inclusion in SLPI ELISAs.

Additionally, proliferative endometrial biopsies (n=5) were cultured with or without progesterone (10^{-6}M) for 24-48hours. After incubation tissue was immediately immersed in Tri reagent for RNA extraction.

5.2.2b Acid extraction of tissue

SLPI is an acid stable molecule with a high isoelectric point (pI) and therefore more soluble in weak acid solution. After 24 hour culture, tissue was acid extracted in the presence of 200µl PBS and 10µl HCl (1N) for 10 minutes. Neutralization was achieved by addition of 10µl NaOH (1N) and 50µl Tris buffer (1M; pH7.2). Supernatant was collected for subsequent inclusion in SLPI ELISAs.

5.2.2c SLPI ELISA

SLPI concentrations were measured in the supernatants from the tissue culture and acid extraction experiments described above. Details of the SLPI ELISA are in section 2.5.2.

5.2.2d Immunohistochemistry

SLPI was localized in endometrium (n=13) and first trimester decidua (n=6) using the protocol detailed in 2.3.6.

5.2.2e RNA extraction and RT-PCR

RNA was extracted from endometrial (n=14), decidual (n=6) and trophoblast (n=3) samples immediately after tissue collection as described in 2.2.1. Similarly, RNA extraction was performed on proliferative endometrial biopsies that had been cultured for 24-48 hours (see above). cDNA was prepared from all samples and SLPI mRNA levels were determined by quantitative PCR using the protocols in 2.2.2 and 2.2.3.

5.2.3 In vitro cell culture studies (cell line)

5.2.3a Cell culture

HeLa and Ishikawa cells were cultured as detailed in 2.4.2. Treatments were as detailed in Table 12. Culture supernatants were collected for inclusion in SLPI ELISAs.

Treatment	Concentration used	Incubation time
Control	N/A	All 24 hours
Progesterone	10^{-6}M	
Dexamethasone	10^{-6}M	
Progesterone + Dexamethasone	Both 10^{-6}M	
Prostaglandin E_2 (PGE_2)	10^{-6}M	
Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)	50ng/ml	
Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)	350ng/ml	
Indomethacin	5μM	
Interleukin-6 (IL-6)	10μg/ml	
LPS	1μg/ml	
Lipoteichoic acid (LTA)	5μg/ml	
PMA	10^{-7}M	
Prolactin	20ng/ml	
Rolipram	10μg/ml	
EGF	0.1μg/ml	

Table 12: Details treatments of HeLa and Ishikawa cell lines. Treatments were all for 24 hours and SLPI secretion into culture medium was subsequently measured by ELISA.

5.2.3b Acid extraction of cells

This was performed as described in section 5.2.2b.

5.2.3c SLPI ELISA

Cell culture supernatants and acid extraction medium were included in SLPI ELISAs as detailed in 2.5.2.

5.2.4 Statistical Analysis

All PCR and ELISA results in this chapter were analyzed by ANOVA. Individual differences were assigned using Fisher's PLSD test. The only exception to this was the experiment shown in Figure 45 (progesterone effect on SLPI mRNA expression in endometrial explants) which was analyzed using a paired t-test.

5.3 Results

5.3.1 Uterine tissue analyses

5.3.1a SLPI secretion by endometrium, decidua and trophoblast

First trimester decidua produces 4.4±1.9 ng SLPI/mg decidua of SLPI. This is significantly higher than production by either endometrium (from throughout the menstrual cycle; $P<0.001$) or trophoblast ($P<0.01$). These tissues produce 0.4±0.1ng/mg and 0.1±0.04ng/mg, respectively (Figure 39). Endometrial SLPI secretion is menstrual cycle dependent and peaks in the late secretory phase. Secretion is 1.2±0.2ng/mg at this time, compared with 0.2±0.1ng/mg in the proliferative phase and 0.3±0.1ng/mg in the early/mid secretory phase (Figure 40; $P<0.0001$). Endometrium from LNG-IUS users secretes 1.4±1.0ng/mg of SLPI and this is comparable to production by late secretory endometrium.

Figure 39: SLPI production by control endometrium (endo; throughout cycle), endometrium from levonorgestrel-intrauterine system users (LNG-IUS), first trimester decidua (dec) and trophoblast (tb). 'n' numbers are shown above bars. Paired letters indicate statistical significance (a :$P<0.001$; b:$P<0.01$).

Figure 40: SLPI production by endometrium from the proliferative (prol), early/mid secretory (ES/MS) and late secretory (LS) phases of the menstrual cycle. Note that the scale of the y-axis differs to that of Figure 39. 'n' numbers are shown above bars. Paired letters indicate statistical significance (a and b:$P<0.0001$).

Figure 39

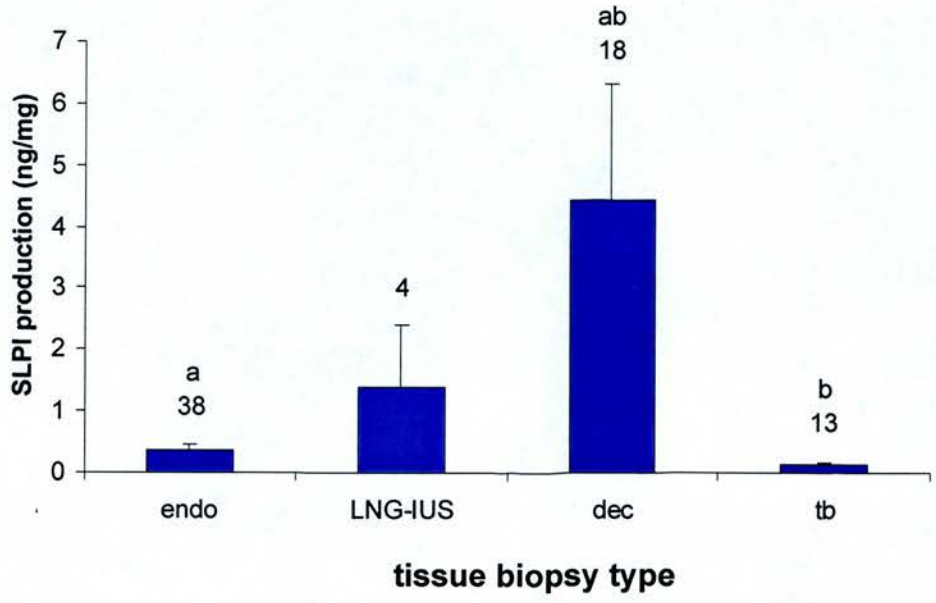
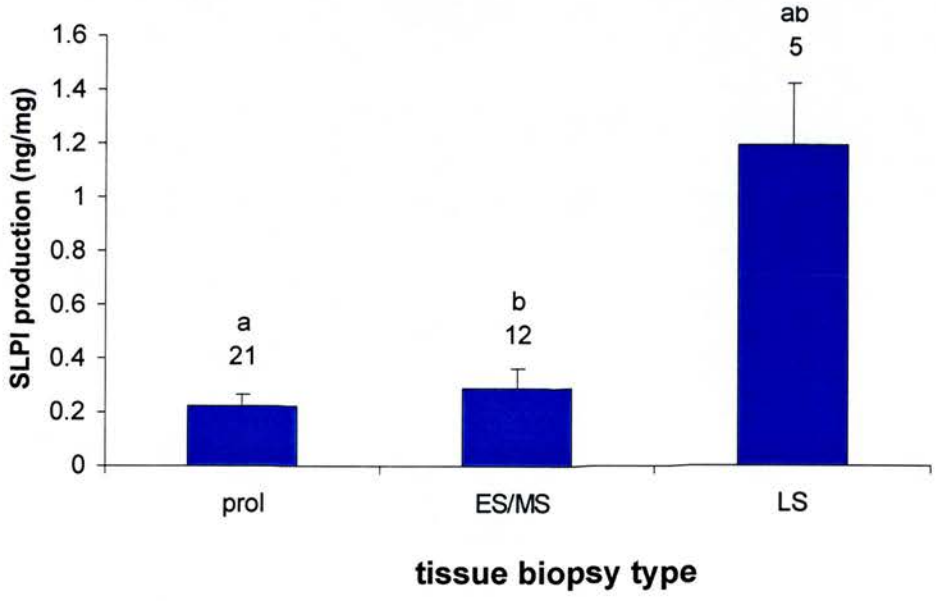


Figure 40



5.3.1b SLPI release from endometrium, decidua and trophoblast during acid extraction

Endometrial release of SLPI was increased by 10.4 fold by acid extraction (relative to release into culture medium). Decidual and trophoblast release was increased by 1.5 and 2.1 fold, respectively (Figure 41; endometrium/decidua $P<0.03$). The result from one decidual sample was discounted from this analysis as the value was 118.1 (i.e. 101 standard deviations from the mean of the other values).

The increased release of SLPI from endometrium due to acid extraction is greatest during the early/mid secretory phase of the menstrual cycle. Release is increased by 19.6 fold at this time while release from proliferative and late secretory endometrium is increased by 5.3 and 2.9 times, respectively (Figure 42: $P<0.01$). These results suggests that the majority of SLPI produced by endometrium in the early/mid secretory phase is not secreted into culture medium.

Figure 41: SLPI release as a result of acid extraction of endometrium (endo), decidua (dec) and trophoblast (tb). SLPI release into acid extracted supernatant is presented as fold increase over secretion into culture medium (y-axis). 'n' numbers are shown above bars. Paired letters indicate statistical significance (a :$P<0.003$).

Figure 42: SLPI release as a result of acid extraction of endometrium from the proliferative (prol), early/mid secretory (ES/MS) and late secretory (LS) phases of the menstrual cycle. SLPI release into acid extracted supernatant is presented as fold increase over secretion into culture medium (y-axis). Note that the scale of the y-axis differs to that of Figure 41. 'n' numbers are shown above bars. Paired letters indicate statistical significance (a :$P<0.001$; b:$P<0.01$).

Figure 41

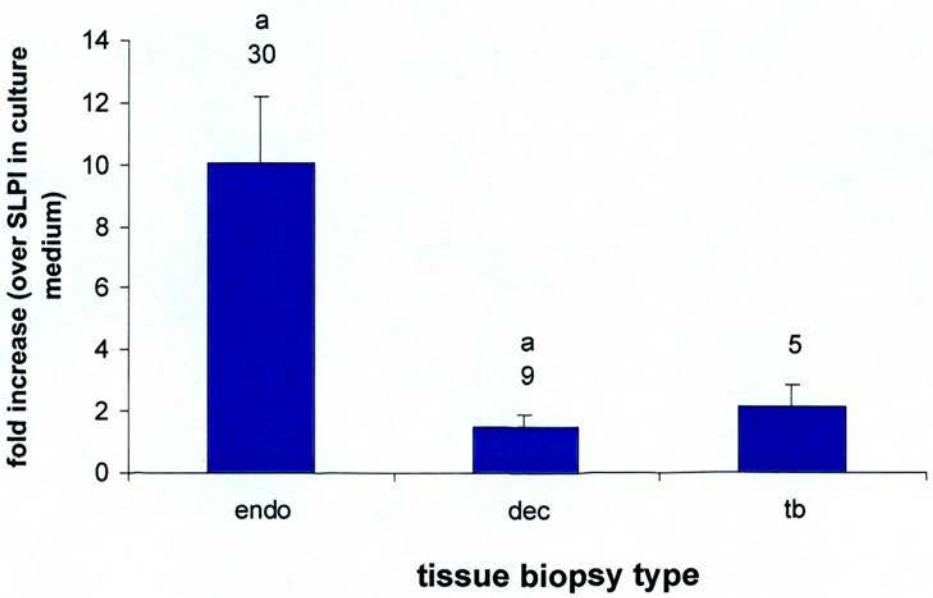
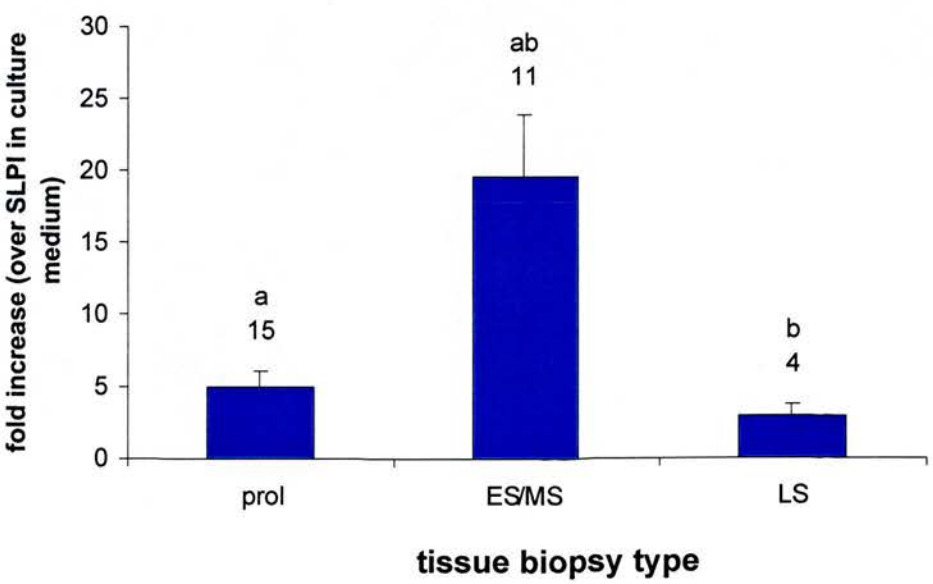


Figure 42

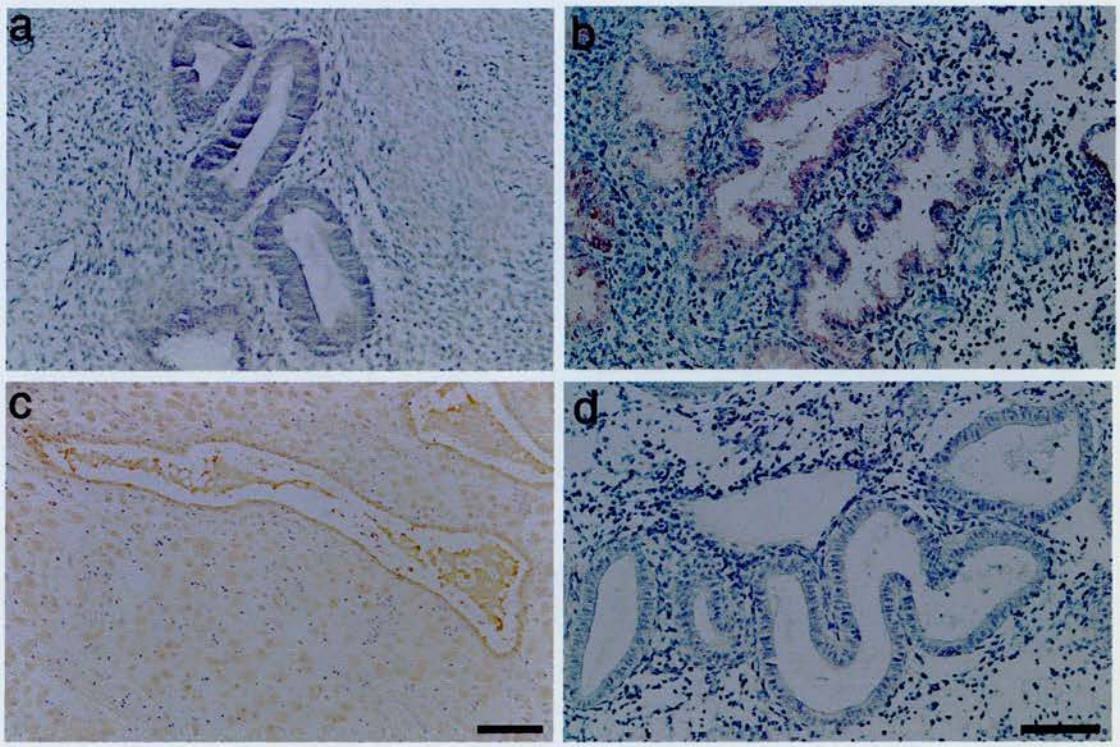


5.3.1c Localization of SLPI in endometrium and decidua

SLPI immunoreactivity was present in the glandular epithelium and secretions in endometrial biopsies from the mid to late secretory phase. In some biopsies positive immunostaining was also observed in the luminal epithelium. SLPI was present mainly in glands in superficial areas of endometrium. No stromal staining occurred (Figure 43b). Very little SLPI immunoreactivity was detected in endometrium from the proliferative and early secretory phase (Figure 43a).

In first trimester decidua SLPI immunoreactivity was again present in glandular epithelial cells and secretions. SLPI was also detected in the decidualized stromal cells of some decidual biopsies (Figure 43c).

Figure 43: Immunohistochemical localization of SLPI in human endometrium and first trimester decidua. **(a).** Proliferative endometrium. No immunoreactivity is present. **(b).** Late secretory endometrium. Immunoreactivity is present in the epithelial cells and secretions of the glands. No immunostaining is observed in the stroma. **(c).** First trimester decidua. SLPI immunoreactivity is present in the glandular epithelium and secretions. Immunostaining is present in stromal cells. **(d).** Negative control. Primary antibody is replaced with goat immunoglobulin at equimolar concentrations. Scale bars = 100μm.


a
b
c
d

5.3.1d SLPI mRNA expression in endometrium and decidua

Decidual samples contained 0.5±0.3 of SLPI mRNA. This was greater than both proliferative and secretory endometrium which had 0.2±0.04 and 0.4±0.3, respectively (Figure 44; not significant).

Progesterone treatment of proliferative endometrial biopsies for 24 or 48 hours resulted in a trend towards increased SLPI mRNA expression (Figure 45; not significant).

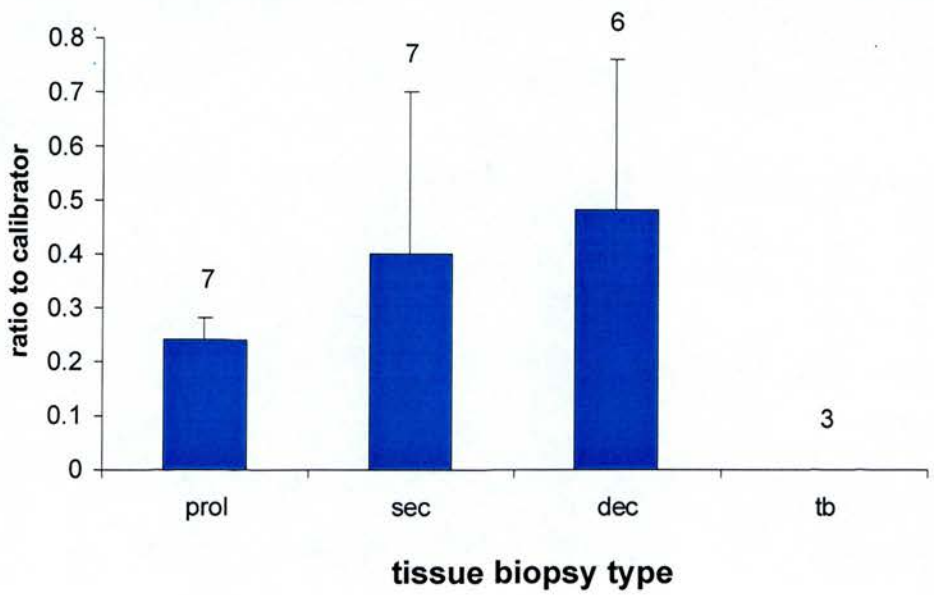


Figure 44: SLPI mRNA expression in endometrium from the proliferative (prol) and secretory (sec) phases of the menstrual cycle, first trimester decidua (dec) and trophoblast (tb) (not significant). All sample PCR measurements are related to an internal control (proliferative endometrial sample) and these ratio are presented in the figure (y axis = ratio to calibrator). 'n' numbers are shown above bars. No significant differences were found between SLPI mRNA expression in different tissue types. It should be noted that trophoblast SLPI mRNA is detectable by PCR and is present at very low levels. The value of less than 0.01 shown in this figure reflects the presence of trophoblast SLPI mRNA relative to an internal control (which expressed SLPI mRNA at a much higher level).

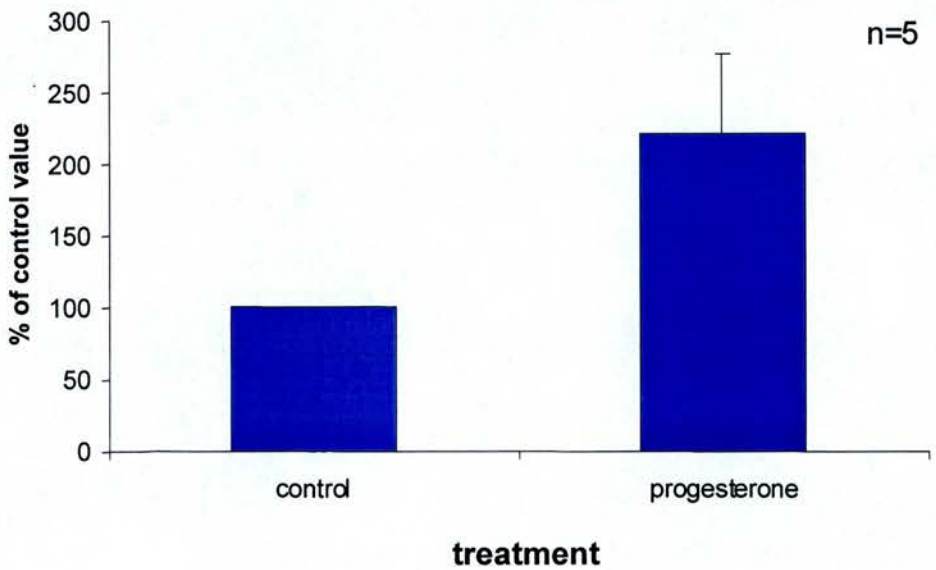


Figure 45: Progesterone effect on SLPI mRNA expression in proliferative endometrium. Data is presented as the % of SLPI mRNA present in control samples. Progesterone treatment resulted in a trend towards increased SLPI mRNA expression in all endometrial explants investigated (n=5; not significant).

5.3.2 *In vitro* cell culture studies (cell lines)

Both the cervical (HeLa) and the endometrial (Ishikawa) epithelial cell lines secreted SLPI under control culture conditions. The cells were treated with various agents (see Table 12) and SLPI in culture supernatants and acid extracted medium was measured by ELISA. No significant regulation of SLPI secretion into culture medium was found for any treatment of HeLa or Ishikawa cells (Figures 46&48). However, when HeLa cells were acid extracted both dexamethasone and EGF treatment resulted in upregulation of SLPI release when compared to control values (Figure 47; $P<0.01$).

Figure 46: SLPI production by the HeLa cervical epithelial cell line. Cells were treated with $PGF_{2\alpha}$ (50ng/ml; PGF2α 50), progesterone (prog), lipopolysaccharide (LPS), lipoteichoic acid (LTA), IL-6, PGE_2, PMA, EGF, indomethacin (indo), rolipram, dexamethasone & progesterone (dex/prog), $PGF_{2\alpha}$ (350ng/ml; PGF2α 350), prolactin and dexamethasone (dex). Values are presented as the % of SLPI released into culture supernatant relative to cells treated under control conditions. 'n' numbers are shown above bars. No significant differences were found to SLPI secretion as a result of different treatments.

Figure 47: SLPI release by HeLa cells when acid extracted. Cells were treated as detailed in Figure 46. Dexamethasone and EGF treatment resulted in increased SLPI release. Values are presented as the % of SLPI released into acid extracted medium relative to cells treated under control conditions. 'n' numbers are shown above bars. Paired letters indicate statistical significance (a and b:$P<0.01$).

Figure 46

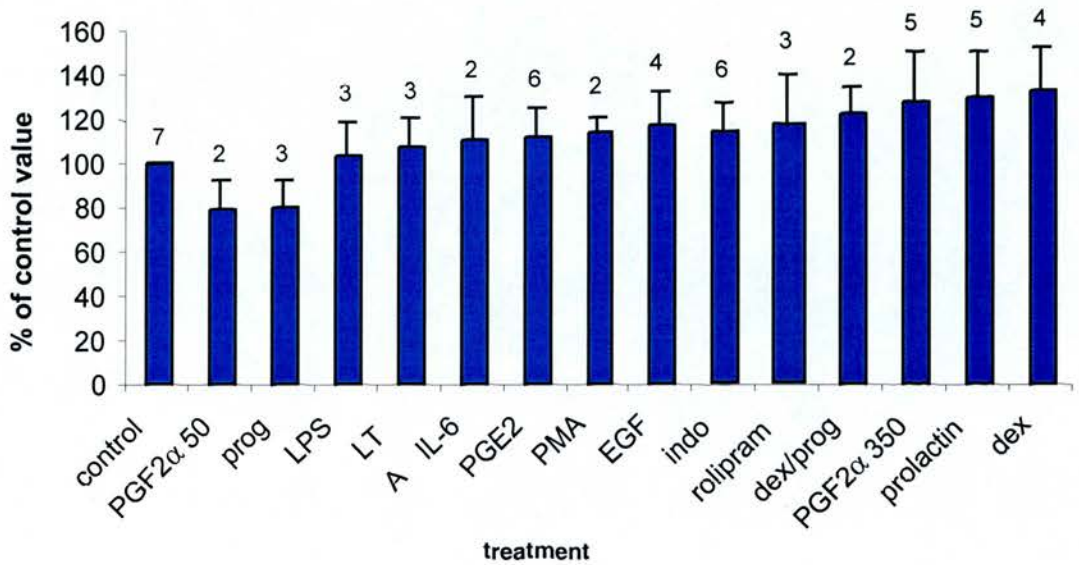
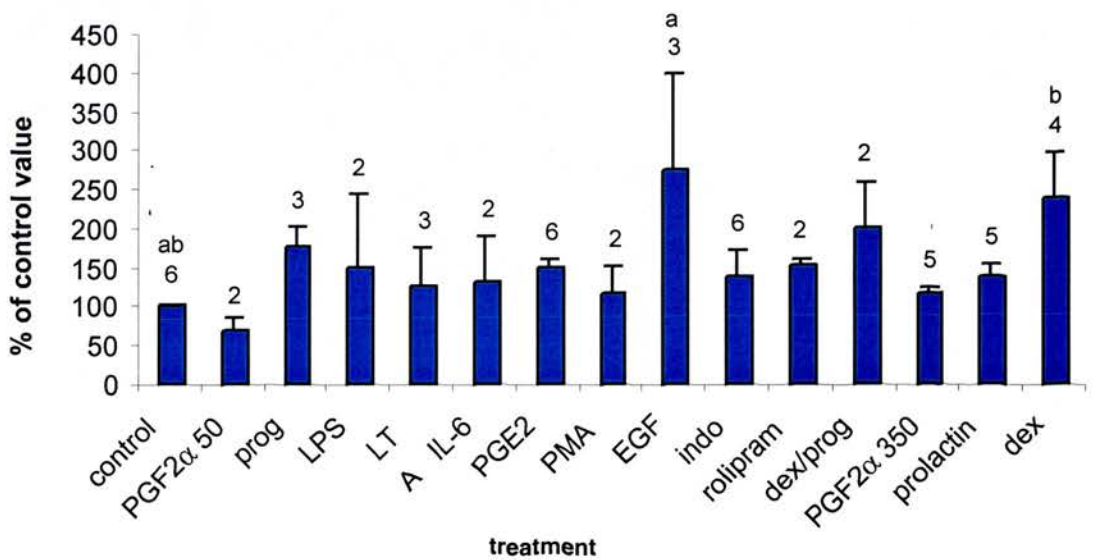


Figure 47



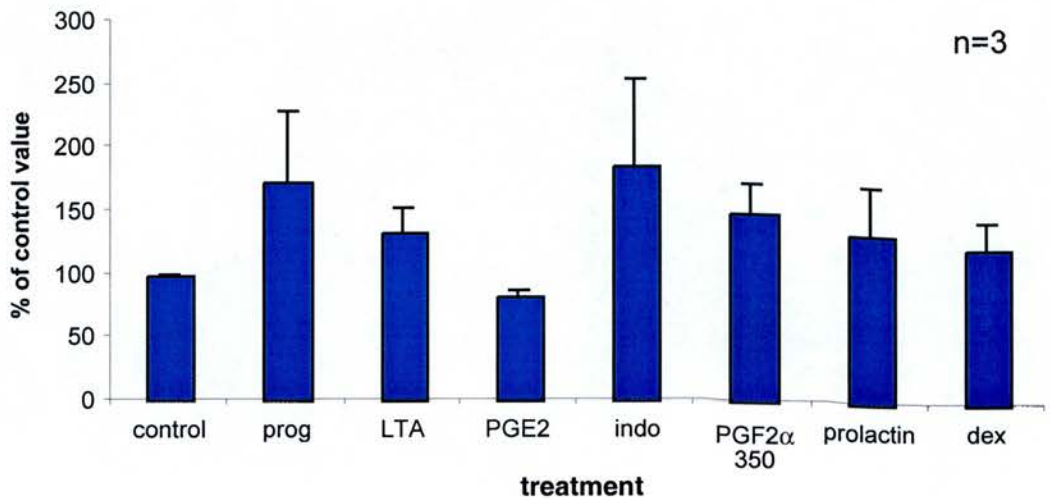


Figure 48: SLPI production by the Ishikawa endometrial epithelial cell line. Cells were treated with progesterone (prog), LTA, IL-6, PGE_2, indomethacin (indo), $PGF_{2\alpha}$ (350ng/ml; PGF2α 350), prolactin and dexamethasone (dex). Results shown are from three separate experiments. Values are presented as the % of SLPI released into culture supernatant relative to cells treated under control conditions. No significant differences were found to SLPI secretion as a result of different treatments.

5.4 Discussion

SLPI, an antibacterial and anti-inflammatory molecule, has been detected in the glandular epithelium of human endometrium and first trimester decidua. This is consistent with the production of SLPI by epithelial cells at other mucosal surfaces such as lung and cervix (Franken, et al., 1989). The detection of SLPI in endometrium is in contrast to previous studies which have failed to detect it in biopsies from both the proliferative and secretory phases of the menstrual cycle (Casslen, et al., 1981; Franken, et al., 1989). SLPI has been reported to be present in uterine fluid although this was thought to be due to diffusion from the cervix (Casslen, et al., 1981). As a result of the work presented here, it now seems likely that endometrial epithelial cells are the principal source of uterine SLPI.

SLPI secretion by endometrium was found to increase in the mid-late secretory phase and this was confirmed by both culture and immunohistochemical studies. This finding may explain why SLPI has not previously been detected in endometrium as in earlier studies very limited numbers of biopsies were investigated (with only two from the secretory phase). Acid extraction was performed to ensure that the SLPI values measured in culture medium were a true representation of SLPI production by the tissue. Interestingly, acid extraction of endometrium resulted in release of SLPI at concentrations far greater than those found in culture medium. This effect was observed mainly during the early-mid secretory phase and suggests that at this time endometrium is producing more SLPI than it is secreting. It may be that initiation of endometrial SLPI production begins after ovulation and that SLPI is then released during the mid-late secretory phase. However, SLPI has been reported to bind to endometrial extracellular matrix proteins (Reed, et al., 1997) and may also remain closely associated with glandular secretory products or the mucosal epithelial cell surface. Acid extraction in *in vitro* experiments may cause release of bound SLPI and reflect more accurately the production of SLPI by endometrium. Decidua, trophoblast and endometrium from other cycle stages released similar amounts of SLPI during culture and acid extraction.

First trimester decidua produced more SLPI than endometrium and trophoblast. SLPI has previously been detected in term decidua in the large decidualized stromal cells (Denison, et al., 1999b) while in early pregnancy decidua SLPI is expressed predominantly in the glandular epithelium with only some stromal cells containing SLPI. This suggests that as pregnancy progresses the site of SLPI expression changes from the glandular epithelium to the stromal cells. A similar change of expression site has been reported in the case of the endothelin B (ET_B) receptor. It is present in the endometrial glandular epithelium becoming apparent in the stromal cells during menstruation and in decidualized stroma (Kohnen, et al., 1998). As pregnancy progresses decidual glands atrophy (Buckley and Fox, 1989) and it may be that the stromal cells begin to produce SLPI in order to maintain decidual function.

SLPI expression is increased in endometrium in the mid-late secretory phase and further, in first trimester decidua. The amounts of mRNA present are consistent with this. This expression profile suggests that SLPI may be regulated by progesterone. The finding that there is a trend towards increased SLPI mRNA expression when proliferative endometrium is treated with progesterone supports this (although this did not reach significance). Also, progesterone has been reported to increase SLPI secretion by cervical explants (Denison, et al., 1999a). Regulation by progesterone could occur either by a direct action on SLPI gene expression or via an indirect mechanism. Progesterone has morphological effects on endometrium such as increasing the glandular surface area and has control over the expression of various inflammatory mediators. These actions may result in secondary effects such as increased SLPI production. Regulation of SLPI expression was investigated in representative cell lines. Progesterone did not cause increased SLPI production by the Ishikawa endometrial epithelial cell line. However, progesterone receptor expression by these cells is controversial (Nishida, et al., 1985; personal communication from Dr Nishida to Dr Kelly, Oct 18, 1994). Co-workers have detected an increase in SLPI mRNA expression in progesterone treated T47D cells (progesterone receptor is expressed at high levels; see Chapter 6, Figure 50). In the HeLa cervical epithelial cell line both dexamethasone and EGF were found to increase SLPI release upon acid extraction. These findings are consistent with

previous reports. Glucocorticoids have been reported to upregulate SLPI production in airway epithelial cells (Abbinante-Nissen, et al., 1995) while EGF treatment of porcine maternal endometrial explants resulted in increased SLPI mRNA expression (although this did not reach significance) (Reed, et al., 1998).

As the increase in SLPI secretion in late secretory endometrium is coincident with decidualization this may contribute to its upregulation. Endometrium from LNG-IUS users is decidualized due to the very high local concentration of progestogen which is 1000 times greater than in serum (Pekonen, et al., 1992). Late secretory endometrium and endometrium from LNG-IUS users also have similar progesterone receptor (PR) expression (Critchley, et al., 1998b). Both have low PR expression (although levels are maintained in the stroma of endometrium from the late secretory phase). Culture results show that endometrium from LNG-IUS users produces similar amounts of SLPI to late secretory endometrium. While this suggests that decidualization may contribute to SLPI production, confirmation is necessary. Immunohistochemical investigation of SLPI in endometrium from LNG-IUS users may clarify this point. Also in the late secretory phase, prior to menstruation, leukocytes infiltrate the endometrium. As neutrophils are known to be a source of SLPI (Bohm, et al., 1992) they may contribute to the increased presence of SLPI in endometrium at this time. However, this is inconsistent with the increased production of SLPI by decidua and also, immunostaining suggests that the glands are the major source of SLPI in endometrium.

There are several potential roles of SLPI in endometrium and decidua. These are summarized in Table 13 and will be discussed in detail in the following text.

Potential role of SLPI in endometrium	Evidence that supports this role	Reference
To limit actions of neutrophils during menstruation	Inhibits neutrophil elastase and cathepsin G	(Thompson and Ohlsson, 1986)
To limit trophoblast invasion	Suggested to maintain uterine-placental border in other species	(Badinga, et al., 1994)
Autocrine growth promoting role	Stimulates DNA synthesis in porcine endometrial epithelial cells	(Badinga, et al., 1999)
Suppression of inflammatory mechanisms	Inhibits NFκB	(Lentsch, et al., 1999)
Antimicrobial actions	Shown to have antimicrobial effects Sequence homology with human β-defensin 1 Endometrial expression profile is similar to defensin 5	(Tomee, et al., 1997) (King, et al., 2000a) (Quayle, et al., 1998)

Table 13: Describes potential roles of secretory leukocyte protease inhibitor (SLPI) in human endometrium.

Neutrophils infiltrate the endometrium prior to menstruation (Poropatich, et al., 1987) and may contribute to tissue breakdown. SLPI is a potent neutrophil elastase inhibitor and may act to limit the actions of neutrophils in endometrium. However, this is unlikely as there is increased expression of SLPI by decidua and neutrophils are rare (King, et al., 1998). Also, SLPI is produced in a superficial location in endometrium and as this is the area sloughed off during menstruation it is unlikely that SLPI is preventing tissue breakdown.

Previously, SLPI has been detected in the maternal endometrium of mammals that have epitheliochorial placentation and it has been suggested that SLPI may maintain the uterine-placental border in these species by controlling trophoblast invasion (Badinga, et al., 1994). The identification of SLPI in human endometrium and decidua shown here indicates that endometrial SLPI is not unique to mammals that have epitheliochorial placentation. Also, a role in control of trophoblast invasion in humans is unlikely as SLPI is found predominantly in the superficial endometrium whereas the trophoblast invades throughout the decidua and into the inner myometrium (Aplin, 1991).

SLPI has also been shown to stimulate DNA synthesis by porcine endometrial epithelial cells suggesting an autocrine growth promoting role (Badinga, et al., 1999). Although auto- and paracrine actions of SLPI are likely to occur in human endometrium, promotion of epithelial cell growth is unlikely as the expression of SLPI does not coincide with the time of maximal epithelial cell growth (i.e. proliferative phase). Receptors for SLPI have been detected in human monocytes although the identity of these is unclear (McNeely, et al., 1997). Further characterization of these binding sites and subsequent localization in endometrium may clarify the potential auto/paracrine roles of SLPI.

Antibacterial, antifungal and antiviral actions of SLPI have been documented (Tomee, et al., 1997). The presence of SLPI in the cervix and male genital tract (high concentrations in semen) has been suggested to offer antimicrobial protection of these mucous membranes (Ohlsson, et al., 1995). A similar role for SLPI in human

endometrium and first trimester decidua is likely. While SLPI may have additional functions in endometrium (as acknowledged in Table 13 and above text) its expression around the time of implantation and during pregnancy suggests that it would be an ideal candidate to offer protection against infection. Intrauterine infection is thought to be the cause of premature labour in at least 20% of cases (Romero, et al., 1989) and natural antibiotics are likely to have a role in controlling this.

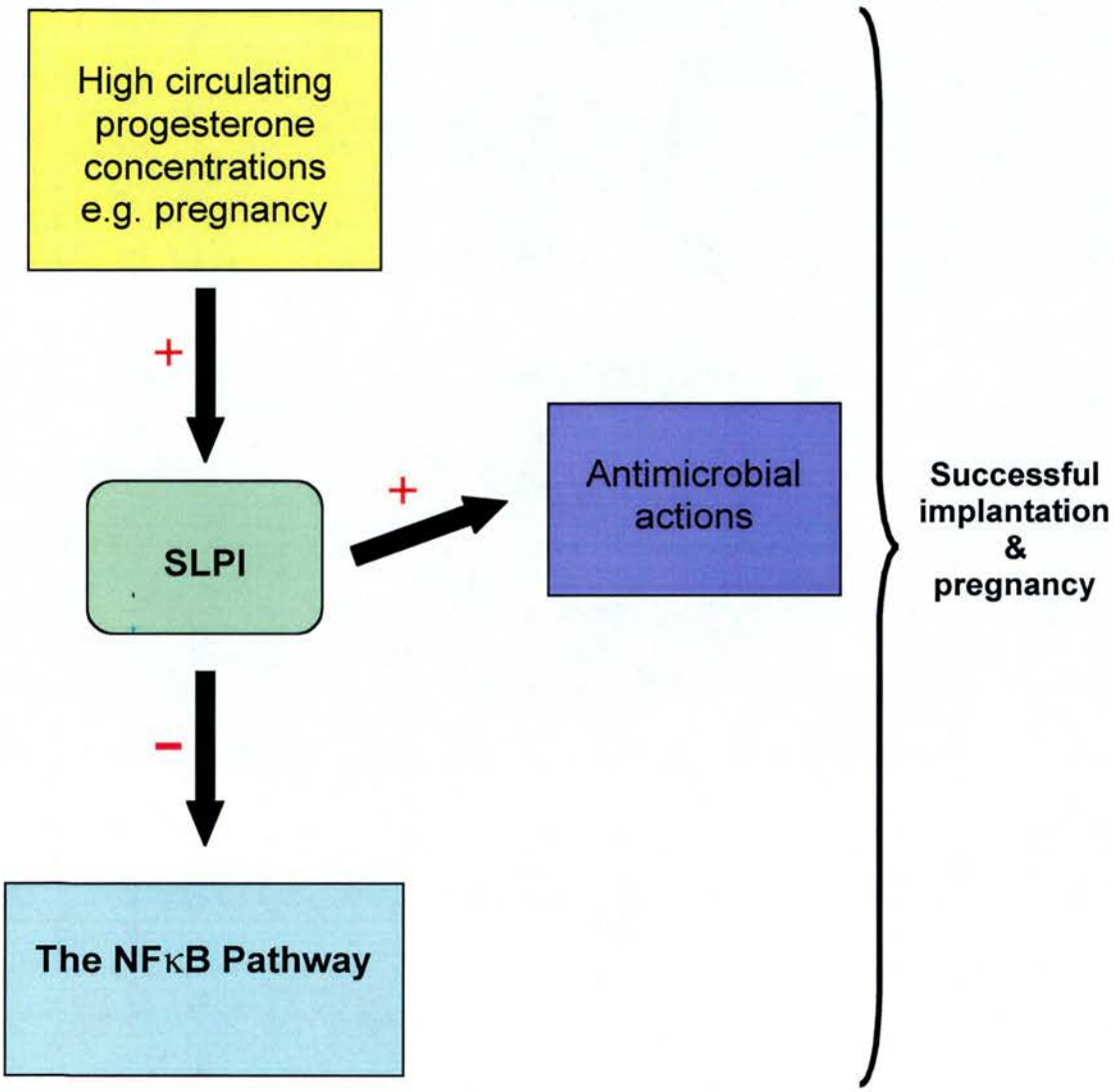
The presence of defensins, which also have antibacterial, antiviral and antifungal properties, has previously been reported in endometrium. Both defensin 5 and β-defensin 1 have been identified in epithelial cells of the endometrium and endocervix (Quayle, et al., 1998; Valore, et al., 1998). Also, defensin 5 has a similar expression profile to that of SLPI with maximal expression in the secretory phase of the menstrual cycle (Quayle, et al., 1998). Interestingly, one area of the SLPI molecule has 37% homology to the defensins and five cysteines are conserved between the molecules (King, et al., 2000a). This area does not coincide with the N terminus of the SLPI molecule, which is the area reported to be responsible for the antibacterial actions. The similar expression profiles and protein sequences of SLPI and the defensins lends further support to an antimicrobial role for SLPI in endometrium.

Several bacteria secrete proteins that degrade SLPI. This suggests that SLPI would otherwise be harmful to these microbes. *Trichomonas vaginalis* is a major pathogen of the lower female genital tract which has been found to secrete cysteine proteases that can degrade recombinant SLPI under assay conditions (Draper, et al., 1998). It is currently unknown if other bacteria, such as those causing sexually transmitted diseases, have evolved virulence factors protecting against SLPI. It has been suggested that the presence of uterine bacteria may be associated with irregular menstrual bleeding (Kristiansen, et al., 1987; Moller, et al., 1995). All patients included in this research project reported regular menstrual cycles and no attempt was made to distinguish between patients with reported normal and heavy menstrual bleeding.

In addition to a possible antimicrobial role, SLPI may have other anti-inflammatory effects in endometrium and decidua. SLPI has been reported to inhibit the NFκB signal transduction pathway via increased levels of IκBβ (Lentsch, et al., 1999). This is particularly interesting given the antibacterial properties of SLPI. IκBβ is thought to be involved in signalling to NFκB only by a small subset of NFκB activators. IκBβ is reported to respond to stimulation by LPS and IL-1 but not by TNF or PMA (Thompson, et al., 1995). LPS is a major component of the cell wall of Gram negative bacteria and as such, is likely to be involved in activation of NFκB during infection. It may be that SLPI acts to limit activation of the pathway by reducing the NFκB response to LPS via increased levels of IκBβ. Also, expression of SLPI has been suggested to be increased by NFκB activation (Nguyen, et al., 1999) and this may form a negative feedback loop. It is possible that SLPI may inhibit the NFκB pathway at multiple levels. However, it is currently unknown if SLPI has any effects on the IKK proteins.

In summary, the antibacterial and anti-inflammatory molecule, SLPI has been detected in human endometrium and decidua. The temporal expression of SLPI suggests a role in implantation and pregnancy. As detailed in previous chapters, progesterone has immunosuppressive actions in the uterus which allow successful pregnancy to occur. The expression of SLPI, along with other antibacterial agents, may act to prevent infection at a time when local immune responses are compromised (Figure 49).

Figure 49: The potential actions of SLPI in human endometrium and first trimester decidua. SLPI is likely to mediate a variety of anti-inflammatory actions. In particular, antimicrobial effects and inhibition of the NFκB signal transduction pathway are likely. Such actions are necessary to prevent inappropriate inflammatory events and to guard against infection at a time when this would be detrimental to the developing fetus.


High circulating progesterone concentrations e.g. pregnancy
+
SLPI
+
Antimicrobial actions
Successful implantation & pregnancy
−
The NFκB Pathway

6. Progesterone control of the NFκB pathway in the T47D cell line

6.1 Introduction

As described in previous chapters the NFκB pathway is involved in the upregulation of genes involved in the inflammatory and immune response (Baldwin, 1996). These include proinflammatory cytokines and adhesion molecules. Glucocorticoids are believed to act as physiological antagonists of NFκB as they suppress the expression of several proinflammatory genes (McKay and Cidlowski, 1999). Glucocorticoids act to inhibit the NFκB pathway and indeed, several proinflammatory molecules that are known to be suppressed by glucocorticoids do not contain glucocorticoid response elements in their promoter regions (McKay and Cidlowski, 1999). This suggests an indirect mechanism of downregulation. Cross-talk between the glucocorticoid and NFκB signal transduction pathways is thought to be involved in this.

Glucocorticoids interact with NFκB at several levels. Dexamethasone has been found to increase expression of the key NFκB inhibitory protein, IκBα (Auphan, et al., 1995; Scheinman, et al., 1995a). Also, the glucocorticoid receptor has been reported to interact directly with the p65 subunit of NFκB (Caldenhoven, et al., 1995; McKay and Cidlowski, 1998; Ray and Prefontaine, 1994; Scheinman, et al., 1995b). This is thought to cause inhibition of the actions of both transcription factors (GR and NFκB). The precise mechanism of this action is unclear and may involve competition for a common cofactor. The sex steroid hormone progesterone, which has immunosuppressive effects in the uterus similar to those of systemic glucocorticoid effects, also suppresses the NFκB pathway. Again, increased IκBα expression and interactions between the progesterone receptor (PR) and NFκB are reported (Kalkhoven, et al., 1996; McKay and Cidlowski, 1998; Wissink, et al., 1998).

Several protein kinases have been implicated in the activation of NFκB. These include IKKα, IKKβ, TBK1, NIK and MEKK1. The effects of progesterone on these kinases are unknown and it is possible that the hormone may interact with the NFκB pathway by modulating expression of one or more of these kinases. Expression of

the scaffolding protein, IKKγ, may also be affected by progesterone. In the context of this research project the most appropriate cell model to study possible effects of progesterone would be an endometrial cell line. However, it is difficult to maintain PR expression in such cells even in the presence of oestradiol. The T47D breast cancer cell line constitutively expresses the PR (Horwitz, et al., 1982) and, as such, provides a good cell model to study progesterone effects on the NFκB pathway in epithelial cells.

Interleukin-10 (IL-10) is an anti-inflammatory cytokine that has been reported to inhibit the NFκB pathway (Lentsch, et al., 1997; Schottelius, et al., 1999). It acts to suppress proinflammatory cytokine production and promotes T helper 2 (Th2) responses (Moore, et al., 1993). Adenosine-3',5'-cyclic monophosphate (cAMP) is also reported to inhibit NFκB and the Th1 response (Chen and Rothenberg, 1994; Kelly, 1994; Manna, et al., 2000; Neumann, et al., 1995). cAMP can also activate NFκB in myeloid cells (Serkkola and Hurme, 1993). The actions of IL-10 and cAMP are relevant to endometrial function as during pregnancy Th2 responses are believed to predominate while Th1 activity is suppressed. The results in this chapter detail the effects of progesterone on the intermediate molecules of the NFκB pathway in T47D cells. The effects of IL-10 and cAMP elevating agents on NIK expression are also reported.

6.2. Methods

6.2.1 *In vitro* cell culture studies (cell line)

6.2.1a Cell culture

Culture of T47D cells was carried out as described in section 2.4.2. MFE and Ishikawa cells (endometrial epithelial) were also cultured under control conditions for 24 hours. Tables 14-16 detail the treatment of the T47D cells. Fetal calf serum stripped of steroid hormones was used throughout.

Treatment	Concentration used	Incubation time
Control	N/A	0, 2, 4, 8, 24 hours
Progesterone	10^{-6}M	2, 4, 8, 24 hours

Table 14: Details the treatment of T47D cells in order to investigate the effects of progesterone on expression of NFκB pathway intermediates over a 0-24 hour timecourse.

Treatment	Concentration used	Incubation time
Control	N/A	0, 0.5, 2, 4, 24 hours
Forskolin	10μM	0.5, 2, 4, 24 hours
PGE_2 + rolipram	10^{-6}M + 1μg/ml	

Table 15: Details the treatment of T47D cells cultured to investigate the effects of forskolin and PGE_2 + rolipram on NIK expression over a 0-24 hour timecourse. Both forskolin and PGE_2 + rolipram are cAMP elevating treatments.

Treatment	Concentration used	Incubation time
Control	N/A	0 and 24 hours
Progesterone	10^{-6}M	24 hours
IL-10	10ng/ml	
Forskolin	10μM	
Forskolin + progesterone	10μM + 10^{-6}M	
Forskolin + IL-10	10μM + 10ng/ml	
PGE2 + rolipram	10^{-6}M + 1μg/ml	
PGE2 + rolipram + progesterone	10^{-6}M + 1μg/ml + 10^{-6}M	
PGE2 + rolipram + IL-10	10^{-6}M + 1μg/ml + 10ng/ml	

Table 16: Details the treatments used to examine the effects of IL-10, progesterone and cAMP elevating agents on NIK mRNA expression.

6.2.1b RNA extraction and RT-PCR

RNA was extracted from T47D, MFE and Ishikawa cells after 24 hour incubations under control conditions. Subsequently, reverse transcription was performed and PCR was used to determine levels of PR mRNA in each of the cell lines. The primers and probe used to measure PR allowed detection of both PR_A and PR_B.

Similarly, after the incubations described in Tables 14-16 RNA was extracted from T47D cells and cDNA was prepared. Messenger RNA expression of the following components of the NFκB pathway was then measured by PCR: IκBα, IKKα, IKKβ, IKKγ, TBK1, NIK and MEKK1. Protocols are described in Chapter 2, section 2.2.

6.2.2 Statistical analysis

The results of the experiment detailed in Table 16 were analyzed by ANOVA. Individual differences were assigned using Fisher's PLSD test.

6.3 Results

6.3a PR mRNA expression in the MFE and Ishikawa endometrial epithelial cell lines compared to the T47D breast epithelial cell line

Figure 50 shows that expression of PR (PR_A+PR_B) mRNA is 1000 fold greater in T47D cells than in either the MFE or Ishikawa endometrial cell lines. This suggests that these endometrial cell lines are unsuitable for the study of progesterone effects on the NFκB pathway and that the T47D cell line is an appropriate cell model for subsequent experiments. The low expression of PR in the endometrial epithelial cell lines may be a result of the cells being of too high passage number. The conditions required to maintain PR expression in these cells have not been well characterized.

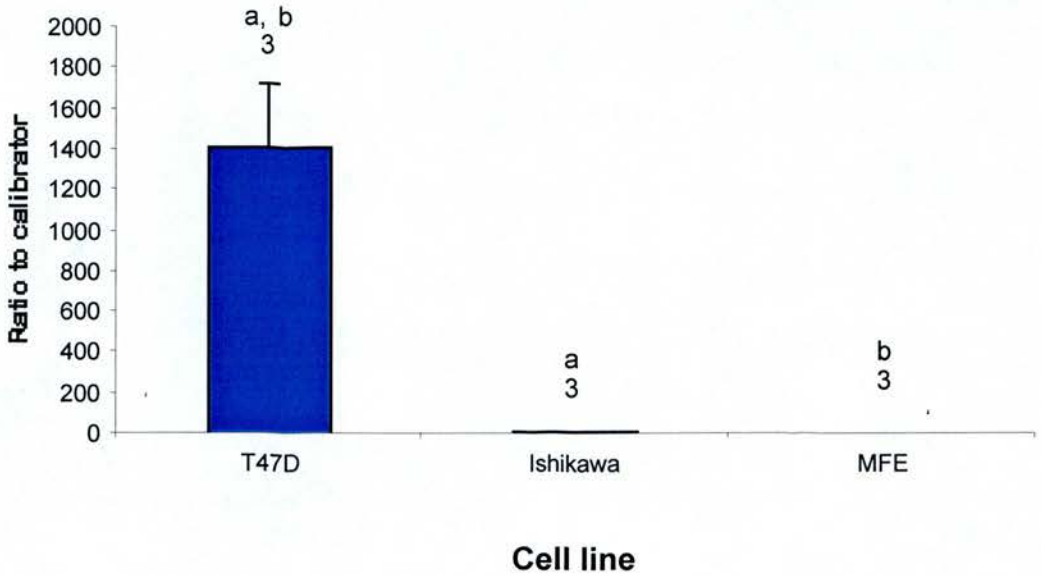


Figure 50: PR (PR_A+PR_B) expression in the T47D breast epithelial cell line and in the Ishikawa and MFE endometrial epithelial cell lines. All PCR measurements are related to an internal control (MFE cell line sample) and the ratio are presented in the figure (y axis = ratio to calibrator). T47D cells express far greater levels of PR than either of the endometrial cell lines. This confirms that the T47D cell line is a suitable cell model for the investigation of progesterone effects on the NFκB pathway. 'n' numbers are shown above bars. Paired letters indicate statistical significance (a and b:$P<0.01$).

6.3b The effects of progesterone on NFκB pathway intermediate expression in T47D cells

Figure 51 shows the effects of progesterone treatment on the following NFκB pathway intermediates: IκBα, IKKα, IKKβ, IKKγ, MEKK1, NIK and TBK1. The results of two separate experiments are shown and data is presented as the ratio of progesterone:control values.

The mRNA expression of IκBα was increased by progesterone at each of the timepoints investigated. Progesterone treatment had little effect on the expression of the other pathway intermediates.

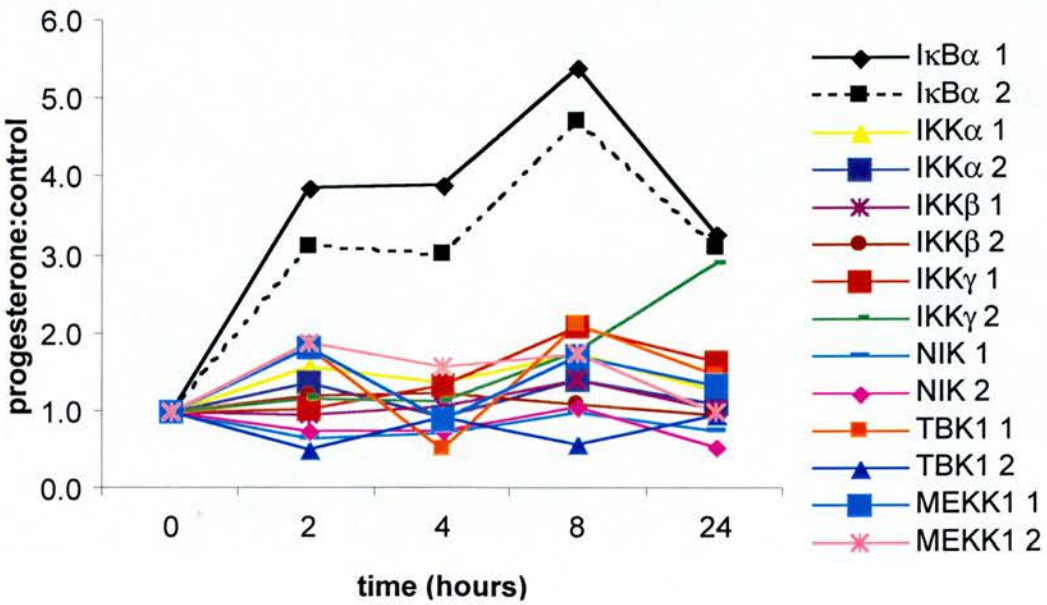


Figure 51: Progesterone effects on NFκB pathway intermediate mRNA expression in T47D cells. Data are presented as the ratio of progesterone:control values (y-axis) at 0, 2, 4, 8 and 24 hours (x-axis). The results of two separate experiments are shown (1 = experiment 1; 2 = experiment 2). IκBα mRNA expression is increased at all timepoints in the presence of progesterone. However, as only two experiments were performed statistical analysis was not possible. Expression of mRNA for IKKα, IKKβ, IKKγ, NIK, MEKK1 and TBK1 is not affected by progesterone at the timepoints investigated.

6.3c The effects of forskolin and PGE_2 + rolipram on NIK mRNA expression over a 24 hour timecourse

This experiment was carried out in order to determine an appropriate incubation time to use in subsequent studies. Throughout the timecourse (0-24 hours) forskolin and PGE_2 + rolipram had little effect on NIK mRNA expression (Figure 52). Hence, 24 hours was chosen as the incubation time for subsequent experiments.

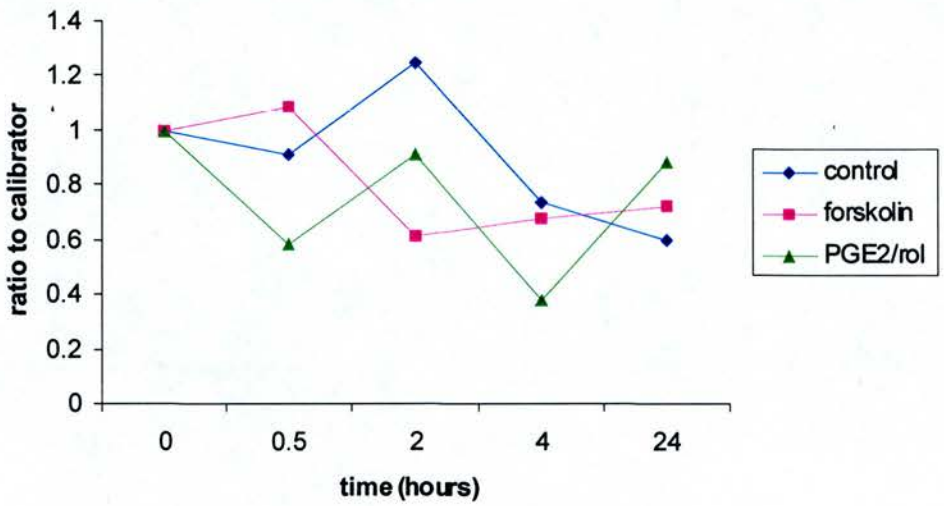


Figure 52: NIK mRNA expression in T47D cells in the presence of cAMP elevating agents. All PCR measurements are related to an internal control (control value at 0hr) and the ratio are presented in the figure (y axis = ratio to calibrator). Treatment with forskolin or rolipram + PGE_2 (PGE2/rol) had no effect on NIK expression at the timepoints investigated.

6.3d The effects of progesterone and IL-10 on NIK mRNA expression in the presence of agents that increase cAMP levels

The results of these experiments were anomalous and as such, the effects of progesterone and IL-10 on NIK mRNA expression remain unclear. The results of three separate experiments suggested that both progesterone and IL-10 reduce NIK expression in the presence of agents that increase cAMP concentrations. However, a further three experiments showed no effect of progesterone (these experiments did not examine the effects of IL-10). Figure 53 shows the mean results of the above six experiments.

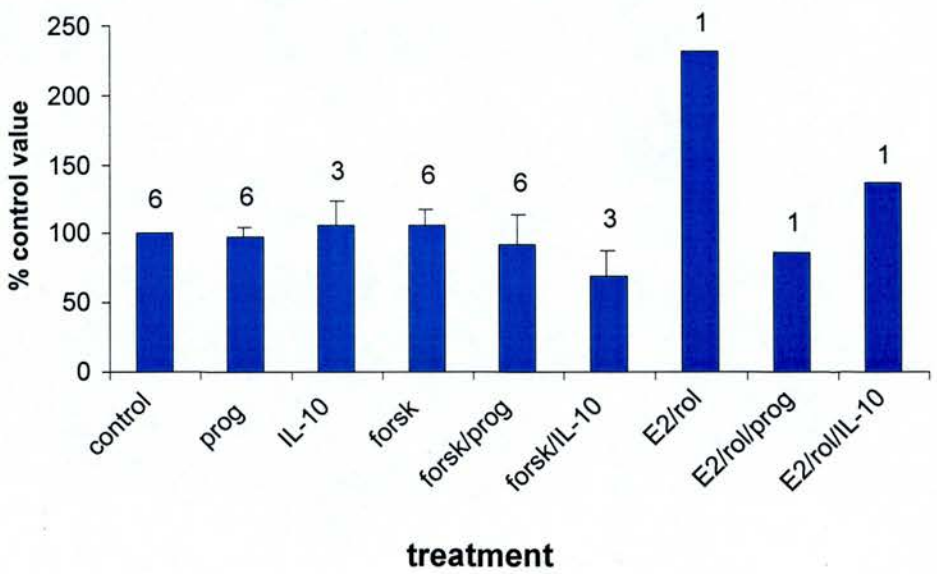


Figure 53: Effects of progesterone and IL-10 on NIK mRNA expression in the absence and presence of cAMP elevating agents. The results shown in this figure suggest that progesterone and IL-10 have no effect on NIK expression (ANOVA: not significant). However, results were anomalous with three experiments showing progesterone and IL-10 to decrease NIK expression in the presence of forskolin while a further three experiments (measuring the effects of progesterone only) showed no effect. Treatments were as follows: control, progesterone (prog), IL-10, forskolin (forsk), forskolin + progesterone (forsk/prog), forskolin + IL-10 (forsk/IL-10), PGE_2 + rolipram (E2/rol), PGE_2 + rolipram + progesterone (E2/rol/prog) and PGE_2 + rolipram + IL-10 (E2/rol/IL-10). 'n' numbers are shown above bars.

6.4. Discussion

The results presented in this chapter detail the actions of progesterone on the NFκB pathway in T47D cells. The T47D cell line has high levels of constitutive PR expression (Horwitz, et al., 1982) and, as shown here, PR mRNA levels are greater than in either the MFE or Ishikawa endometrial epithelial cell lines. Thus, the T47D cell line was used for the studies described in this chapter. Activation of the NFκB pathway is primarily controlled by phosphorylation-dephosphorylation events and this is likely to be crucial to efficient activation of the pathway during the immune response to infection. However, the pathway is also involved in physiological events such as cell differentiation and it may be that in these circumstances levels of the pathway intermediates are modulated. Progesterone is maintained at high levels throughout the secretory phase of the menstrual cycle and during pregnancy and so, any effects on the NFκB pathway are unlikely to be acute. Therefore, in the T47D cell model progesterone effects on NFκB intermediates were investigated over a 0-24 hour timecourse. Progesterone was found to increase IκBα mRNA expression which would result in the inhibition of NFκB activation. This confirms the results of previous studies (Miller and Hunt, 1998; Wissink, et al., 1998). Progesterone was not found to alter the levels of mRNA for the kinases, IKKα, IKKβ, MEKK1, NIK and TBK1, or the scaffolding protein, IKKγ. It is possible that lengthened exposure to progesterone may affect these kinases or that their expression/activity is altered by an indirect mechanism.

The effects of progesterone and interleukin-10 (IL-10) (in the presence of cAMP elevating agents) on NIK expression were also investigated. IL-10 is an anti-inflammatory cytokine. Its actions include suppressing the production of cytokines such as IL-1, IL-6 and IL-8 and diminution of the T helper 1 (Th1) response (Moore, et al., 1993). IL-10 inhibits NFκB activity and is reported to achieve this by initially inhibiting IKK activity and, after prolonged treatment, via inhibition of DNA binding of NFκB (Schottelius, et al., 1999). Additionally, preservation of IκBα protein expression has been reported and it was suggested that this was due to induction of gene expression or stabilization of mRNA (Lentsch, et al., 1997). cAMP is involved

in signalling to protein kinase A as a result of activation by many mediators e.g. PGE. As for IL-10, cAMP has been reported to inhibit the NFκB pathway. This inhibition has been suggested to involve downregulation of NFκB, upregulation of IκBα expression and inhibition of IκBα phosphorylation (Chen and Rothenberg, 1994; Manna, et al., 2000; Neumann, et al., 1995). It has also been reported that cAMP activates NFκB (Serkkola and Hurme, 1993) in myeloid cells although this appears to depend on the maturity of the cells.

The effects of both IL-10 and cAMP on the NFκB pathway are relevant to endometrial physiology. For example, in pregnancy it is believed that maternal cell mediated immunity is suppressed allowing humoral immunity to dominate. This is thought to involve an increased T helper 2 (Th2) cell activity with a simultaneous decrease in Th1 activity. This hypothesis was based on studies in the mouse (Wegmann, et al., 1993) but relevance to man has been assumed. Several cytokines are involved in this bias towards Th2 activity and IL-10 is likely to be a crucial contributor (via its Th1 suppressing actions). PGE, via stimulation of cAMP, is also involved in suppressing the Th1 response (Kelly, 1994). Interestingly, inhibition of NFκB has been found to impair Th1 but not Th2 activity (Aronica, et al., 1999). Thus, there is scope for interactions between progesterone, cAMP, IL-10 and the NFκB pathway.

The effects of progesterone and IL-10 on NIK mRNA expression in the presence of cAMP elevating agents are unclear. The results of three consecutive experiments showed, on average, a 40% and 50% reduction in NIK mRNA levels as a result of treatment (in the presence of forskolin) with IL-10 and progesterone, respectively. However, a further three experiments showed no modulation of NIK mRNA expression by progesterone. The reasons for these disparate findings are unclear. However, the original three experiments coincided with a time when the T47D cells were not growing well. The latter experiments (i.e. those that showed no effect) were performed at a time when cell growth was normal. This suggests that stage of cell cycle or some other factor relating to rate of cell growth may have been responsible. The effects of progesterone and IL-10 suggest cross-talk between the cAMP-protein

kinase A signal transduction pathway and others. For example, it has been reported that the presence of 8-bromo-cAMP can potentiate the actions of the progesterone receptor. The progesterone antagonist RU486 has the actions of a partial agonist in the presence of 8-bromo-cAMP (Beck, et al., 1993). The reason for this is thought to be due to differential recruitment of the corepressors, nuclear receptor corepressor (NCoR) and silencing mediator for retinoid and thyroid hormone receptor (SMRT). The presence of 8-bromo-cAMP reduces the interaction of NCoR and SMRT with the PR thus potentiating its transcriptional activity (Wagner, et al., 1998). One possible mechanism for the results described in experiment 3 is that the reduction in NIK mRNA by progesterone is due to forskolin potentiating the actions of PR. There are also possible interactions between IL-10 and cAMP. For example, cAMP has been found to increase IL-10 synthesis (Eigler, et al., 1998; Strassmann, et al., 1994).

In summary, the results detailed above confirm that progesterone increases IκBα mRNA expression and is likely to inhibit NFκB via this mechanism. There was no evidence for actions of progesterone on the upstream kinases in the NFκB pathway. It is possible that progesterone and IL-10 suppress NIK mRNA expression in the presence of cAMP elevating agents. Unfortunately, the results presented here are inconclusive and confirmation is necessary.

7: General Discussion

7.1 Synopsis of results

This thesis presents evidence suggesting that the proinflammatory NFκB signalling pathway is involved in endometrial function. Expression of NFκB signalling intermediates was investigated along with that of the NFκB activator, CD40, and the NFκB inhibitor, SLPI. Progesterone control of the NFκB pathway was also studied.

As detailed previously, activation of the NFκB pathway has been shown to involve several protein kinases: IKKα, IKKβ, NIK, MEKK1 and TBK1 (see Chapter 1, Figure 4). IKKα and IKKβ are held in a complex that also contains a scaffolding protein, IKKγ. The result of activation of these kinases is the phosphorylation and subsequent degradation of the endogenous inhibitor, IκBα. This allows translocation of NFκB to the nucleus where activation of gene expression occurs. The expression of the above intermediates was investigated in endometrium and in T47D cells.

Expression profiles of NFκB pathway intermediates were investigated in endometrium and first trimester decidua. IκBα and TBK1 mRNA expression was increased in endometrium from the perimenstrual phase suggesting activation of the pathway as a result of progesterone withdrawal. This is consistent with reports which describe inhibitory actions of progesterone on the NFκB pathway (Kalkhoven, et al., 1996; McKay and Cidlowski, 1998; Wissink, et al., 1998). As a result of this, progesterone withdrawal would be expected to release the inhibition, allowing NFκB activation.

In first trimester decidua, differential expression of intermediates was observed suggesting that the high progesterone concentrations of early pregnancy may influence signalling to NFκB. The proinflammatory signalling pathway (MEKK1-IKKβ) to NFκB was downregulated in decidua while there was increased expression of NIK and IKKα. These kinases are thought to be involved in morphogenic signalling to NFκB (Hu, et al., 1999; Takeda, et al., 1999). The expression of NIK and IKKα in endometrium and decidua was examined by immunolocalization. Both

proteins were expressed primarily in the glandular epithelium and the endothelium of endometrium. In decidua, immunoreactivity was also detected in the decidualized stromal cells. Additionally, NFκB activity was investigated in an endometrial epithelial cell line. The presence of NFκB inhibitors resulted in an unexpected increase in COX-2 and IL-8 expression. It should be noted that glucocorticoids regulate the NFκB pathway in sites other than the uterus and may not have the same effect as progesterone on pathway intermediates. Also, other transcription factors (such as AP-1) are known to interact with NFκB and these are likely to modulate its actions in the uterus.

The NFκB activating, CD40-CD40 ligand system was investigated in several reproductive tissues. CD40 was found to be expressed predominantly in the perivascular cells of endometrium and decidua (diffuse stromal staining was also present in decidua). Similar expression was observed in myometrium and cervix. CD40 expression was found to be progesterone independent as there were no changes in expression during the menstrual cycle or in T47D cells treated with progesterone. In endometrium, Thy-1 (expressed by fibroblasts) was also found to be present in the perivascular cells. This finding is consistent with previous reports of Thy-1 expression in these cells (Oliver, et al., 1999) and suggests that the source of CD40 expression in endometrium is likely to be myofibroblasts (the earlier study also detected α-smooth muscle actin expression). It was not possible to measure CD40L protein expression in the current study. However, investigation of mRNA expression indicated that both CD40 and CD40L are present at higher levels in decidua than in endometrium.

SLPI is a neutrophil elastase inhibitor that also has antimicrobial activity and inhibits NFκB. This anti-inflammatory protein was found to be expressed by endometrium during the mid-late secretory phase of the menstrual cycle. Secretion was increased in first trimester decidua. Acid extraction studies indicated that endometrium, cultured in *in vitro* studies, may not release all of the SLPI which is produced into culture medium. SLPI was detected immunohistochemically in the glandular epithelium of endometrium and decidua and was also expressed in the decidualized

stromal cells of some first trimester decidual biopsies. These results suggested that SLPI expression may be under progesterone control. This was confirmed by the finding that culture of proliferative endometrial explants in the presence of progesterone resulted in increased SLPI mRNA expression. This is consistent with a colleague's results showing that progesterone has been found to increase SLPI mRNA levels in the progesterone receptor expressing T47D cell line. Regulation of SLPI expression was also investigated in cell lines. Dexamethasone and EGF were found to increase SLPI production by HeLa cells.

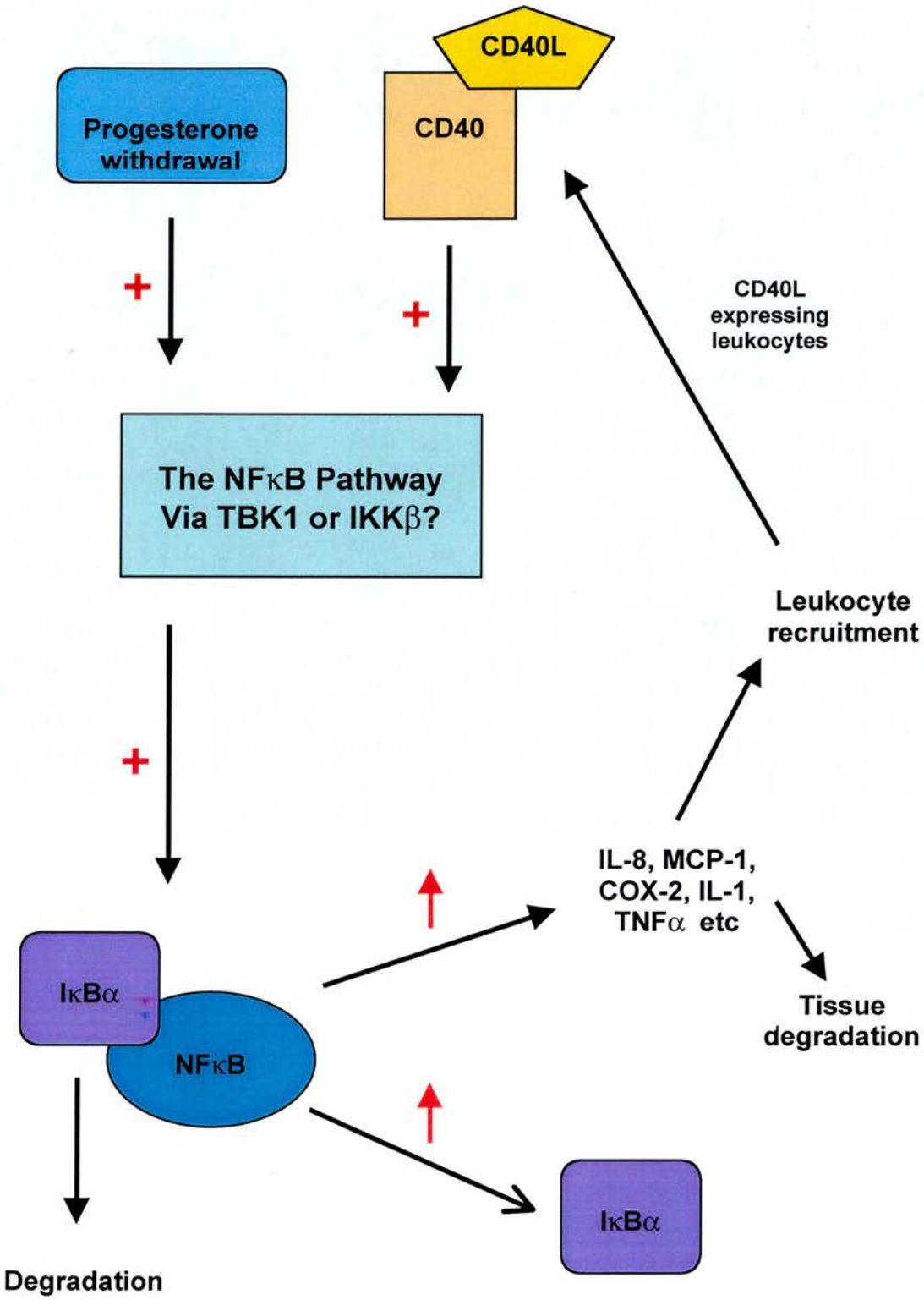
Progesterone regulation of the NFκB pathway was studied in T47D cells. These cells express the progesterone receptor at far greater levels than the endometrial cell lines investigated (MFE and Ishikawa) and as such, are an appropriate cell model for the study of progesterone effects on the NFκB pathway. In keeping with previous studies (Miller and Hunt, 1998; Wissink, et al., 1998), progesterone was found to upregulate IκBα mRNA expression. This is thought to inhibit NFκB and is likely to occur as a direct result of increased transcriptional activity. Expression of the protein kinases and IKKγ was not affected by progesterone during the 24 hour timecourse. However, the results suggest that in the presence of cAMP elevating agents, progesterone (and IL-10) reduces mRNA expression of NIK. Therefore, it is possible that, under circumstances where cAMP levels are raised, progesterone may inhibit NFκB activity via this mechanism. However, the results presented are inconclusive and further investigation is necessary. The following text discusses the results in the context of menstruation and early pregnancy.

7.2 Regulation of inflammatory mediators during menstruation

Menstruation is characterized by an inflammatory like reaction which involves increased inflammatory mediator expression, leukocyte infiltration and tissue oedema (Kelly, 1994). The NFκB pathway regulates the expression of several inflammatory molecules which are involved in menstruation e.g. IL-8, COX-2 (Adcock, et al., 1997; Mukaida, et al., 1990). The results presented in this thesis support a role for the NFκB pathway in the events associated with menstruation. The increased expression of the endogenous inhibitor, IκBα, in the perimenstrual phase suggests that NFκB is activated at this time. Premenstrual progesterone withdrawal will activate NFκB causing upregulation of IκBα. Also, the IKK-like kinase, TBK1, is upregulated in the perimenstrual phase indicating that this kinase may be involved in signalling to NFκB during menstruation. Activation of NFκB prior to, and during, menstruation is likely to contribute to the upregulation of inflammatory mediator expression which has been reported to occur at this time. In turn, this will mediate events such as leukocyte recruitment and tissue degradation.

The results described in this thesis suggest that the CD40-CD40L system may contribute to NFκB activation during menstruation. The CD40-CD40L system is likely to be involved in mediating interactions between resident structural cells and infiltrating leukocytes (Smith, et al., 1997). Such leukocytes are a likely source of CD40L in endometrium. CD40L has also been reported to be produced by platelets (Henn, et al., 1998). Previously, it has been shown that CD40 engagement increases IL-8 production and COX-2 expression in fibroblasts (Sempowski, et al., 1997a; Sempowski, et al., 1998; Zhang, et al., 1998). The expression of CD40 in the endometrial perivascular region is consistent with a role in the upregulation of IL-8, COX-2 and MCP-1 which occurs in this area premenstrually (Critchley, et al., 1999; Jones, et al., 1997). Regulation of these mediators may occur by CD40 activation of the NFκB pathway and will contribute to leukocyte recruitment. A role in endometrial repair processes during menstruation is also likely as leukocytes are known to be involved in this (Salamonsen, 2000). CD40L-expressing leukocytes may activate CD40 present on endometrial myofibroblasts. Myofibroblasts are involved

in wound healing (Schurch, et al., 1992) and therefore it is possible that activation of these cells could contribute to the repair mechanisms involved in the final stages of menstruation. The contribution of the NFκB pathway and CD40 to events associated with menstruation are summarized in Figure 54.

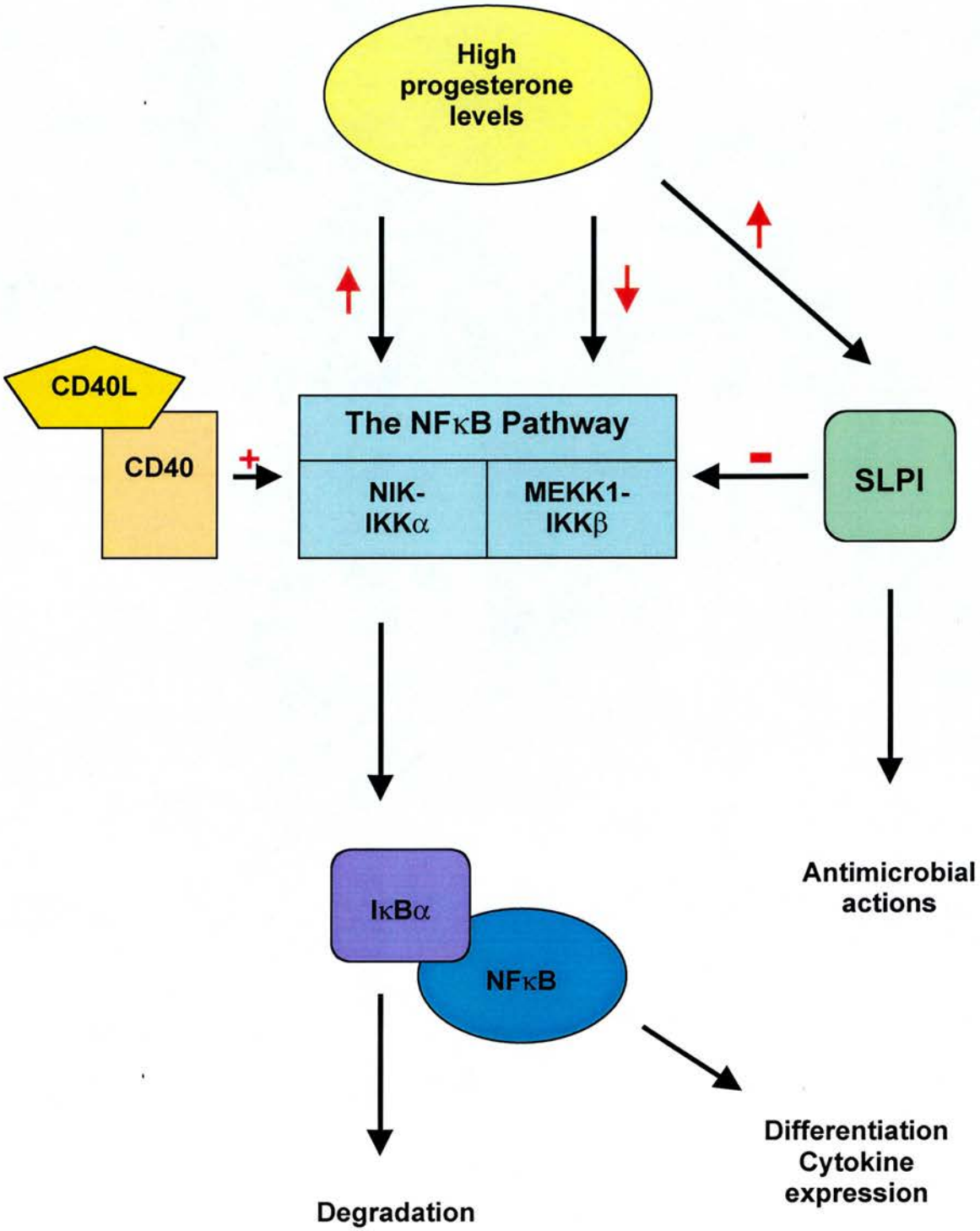

CD40L
CD40
Progesterone withdrawal
+
+
CD40L expressing leukocytes
The NFκB Pathway Via TBK1 or IKKβ?
Leukocyte recruitment
+
IL-8, MCP-1, COX-2, IL-1, TNFα etc
IκBα
NFκB
Tissue degradation
IκBα
Degradation

7.3 Regulation of inflammatory mediators in early pregnancy

High progesterone levels prevail in pregnancy resulting in local immunosuppression and anti-inflammatory actions (Siiteri and Stites, 1982). Consistent with this, the results presented here indicate the downregulation of proinflammatory signalling to NFκB in early pregnancy. This is demonstrated by the decreased mRNA expression of MEKK1 and IKKβ (IKKβ mediates inflammatory signals). However, in contrast there is upregulation of NIK and IKKα expression. IKKα has been reported to be involved in morphogenic signalling to NFκB in mice although the implications of this are unclear. In decidua, NIK and IKKα may be involved in the differentiation events that occur as decidualization is completed during early pregnancy. Alternatively, involvement in regulation of mediator expression (e.g. COX-2) in decidua is possible. In addition to the upregulation of the NIK-IKKα pathway in decidua there is also increased mRNA expression of CD40 and CD40L. This system may have a similar role to that of NIK-IKKα and indeed, may activate this pathway. Also, as there is a large number of lymphoid cells in first trimester decidua (Loke and King, 1995) CD40 may be involved in interactions between these cells and decidual stromal cells.

SLPI may also contribute to the anti-inflammatory processes associated with pregnancy. SLPI expression is apparent in endometrium at the time of implantation and in first trimester decidua and its expression is under progesterone control. SLPI has been reported to have antimicrobial activity and this suggests that it may be part of the innate defence against infection that is crucial to successful pregnancy. Both the temporal and spatial expression profile of SLPI is consistent with such a role. SLPI is also likely to contribute to suppression of proinflammatory mediator expression via inhibitory actions on the NFκB pathway. It is acknowledged that there are additional possible roles for SLPI in endometrium including inhibition of neutrophil actions, control of trophoblast invasion and auto/paracrine effects. However, the data presented in this thesis is most consistent with a protective role during pregnancy. The roles of the NFκB pathway, CD40 and SLPI in early pregnancy are detailed in Figure 55.

Figure 55: The role of the NFκB pathway, CD40-CD40L system and SLPI in early pregnancy. The intermediates in the NFκB pathway are differentially regulated in first trimester decidua. The proinflammatory signalling pathway to NFκB (MEKK-1-IKKβ) is downregulated while mediators thought to be involved in morphogenesis (NIK-IKKα) are increased. This suggests that NFκB may have a role in controlling expression of mediators that are crucial to pregnancy and also, those involved in growth and differentiation. The CD40-CD40L system may contribute to this by activating NFκB via NIK and IKKα. SLPI is an anti-inflammatory molecule that has an expression profile consistent with an antimicrobial role in pregnancy. It may also inhibit the NFκB pathway.


High progesterone levels
CD40L
CD40
+
The NFκB Pathway
NIK-IKKα
MEKK1-IKKβ
-
SLPI
IκBα
NFκB
Antimicrobial actions
Differentiation
Cytokine expression
Degradation

7.4 Suggestions for Future Study

1. The current study details the effects of progesterone on the NFκB pathway in a breast cancer epithelial cell line and examines the pathway in whole endometrial and decidual biopsies. However, as detailed earlier, the endometrial perivascular cells are the predominant site of progesterone receptor expression in the late secretory phase of the cycle (Wang, et al., 1998). This suggests that, if NFκB activation occurs as a direct result of premenstrual progesterone withdrawal, it is these cells which will be most affected. Several questions should be addressed. Which NFκB pathway intermediates are expressed by the perivascular cells? Are these intermediates regulated by progesterone? Is NFκB activated in these cells premenstrually?

2. CD40 was shown to be present on the endometrial perivascular cells. Previous studies have suggested that these cells are of myofibroblast lineage and that they express COX-2, IL-8 and MCP-1 (Critchley, et al., 1999; Jones, et al., 1997; Oliver, et al., 1999). Are CD40, myofibroblast markers and these inflammatory mediators coexpressed in the same cells? If so, does engagement of CD40 on these cells result in increased expression of COX-2, IL-8 and MCP-1? Does CD40 activate NFκB in the perivascular cells and is this responsible for the upregulation of mediators?

3. The localization of CD40L expressing cells in endometrium was not possible during this study. However, the identification of such cells in endometrium and other reproductive tissues is crucial to our understanding of the role of the CD40-CD40L system. Which cell types express CD40L?

4. This study has suggested that SLPI has an antimicrobial and anti-inflammatory role in endometrium and decidua. SLPI binding sites have been detected in monocytes (McNeely, et al., 1997). Are similar binding sites present in endometrium and decidua? What are the results of the interaction of SLPI with these sites?

5. Similar to menstruation, parturition and cervical ripening are associated with inflammatory responses. Both are characterized by increased expression of inflammatory mediators and leukocyte infiltration. This suggests that NFκB activation may also be involved in these reproductive events. Indeed, it has been reported that the nuclear localization of the p65 subunit of NFκB increases with advancing gestation (Gibb, et al., 2000). Also, the finding that CD40 is expressed by both myometrium and cervix suggests that this system is present and may contribute to activation of NFκB. Are the various NFκB pathway intermediates expressed by cervix, myometrium, term decidua and fetal membranes and in which cell types? Are any of the intermediates upregulated immediately prior to, or during, parturition or in the case of infections associated with preterm labour?

6. Pathophysiological reproductive events are also likely to involve the NFκB pathway and systems that interact with it. For example, menstrual problems such as menorrhagia and dysmenorrhea are associated with abnormal prostaglandin production. Is there aberrant activation of the NFκB pathway or abnormal expression of the intermediates in these conditions? Similarly, are CD40 or CD40L expressed?

Bibliography

Abberton, K. M., Healy, D. L. and Rogers, P. A. (1999) Smooth muscle alpha actin and myosin heavy chain expression in the vascular smooth muscle cells surrounding human endometrial arterioles. *Hum Reprod*, **14**, 3095-100

Abbinante-Nissen, J. M., Simpson, L. G. and Leikauf, G. D. (1995) Corticosteroids increase secretory leukocyte protease inhibitor transcript levels in airway epithelial cells. *Am J Physiol*, **268**, L601-6

Abe, T., Kobayashi, N., Yoshimura, K., et al. (1991) Expression of the secretory leukoprotease inhibitor gene in epithelial cells. *J Clin Invest*, **87**, 2207-2215

Abel, M. H. and Baird, D. T. (1980) The effect of 17 beta-estradiol and progesterone on prostaglandin production by human endometrium maintained in organ culture. *Endocrinology*, **106**, 1599-606

Adcock, I. M., Newton, R. and Barnes, P. J. (1997) NF-kappa B involvement in IL-1 beta-induction of GM-CSF and COX-2: inhibition by glucocorticoids does not require I-kappa B. *Biochem Soc Trans*, **25**, 154S

Akiyama, M., Okabe, H., Takakura, K., et al. (1999) Expression of macrophage inflammatory protein-1alpha (MIP-1alpha) in human endometrium throughout the menstrual cycle. *Br J Obstet Gynaecol*, **106**, 725-30

Alderson, M. R., Armitage, R. J., Tough, T. W., et al. (1993) CD40 expression by human monocytes: regulation by cytokines and activation of monocytes by the ligand for CD40. *J Exp Med*, **178**, 669-74

Allavena, P., Bianchi, G., Zhou, D., et al. (1994) Induction of natural killer cell migration by monocyte chemotactic protein-1, -2 and -3. *Eur J Immunol*, **24**, 3233-6

Altenburg, A., Baldus, S. E., Smola, H., et al. (1999) CD40 ligand-CD40 interaction induces chemokines in cervical carcinoma cells in synergism with IFN-gamma. *J Immunol*, **162**, 4140-7

Aplin, J. D. (1991) Implantation, trophoblast differentiation and haemochorial placentation: mechanistic evidence in vivo and in vitro. *J Cell Sci*, **99**, 681-92

Arici, A., Head, J. R., MacDonald, P. C., et al. (1993) Regulation of interleukin-8 gene expression in human endometrial cells in culture. *Mol Cell Endocrinol*, **94**, 195-204

Arici, A., MacDonald, P. C. and Casey, M. L. (1995) Regulation of monocyte chemotactic protein-1 gene expression in human endometrial cells in cultures. *Mol Cell Endocrinol*, **107**, 189-97

Arici, A., Seli, E., Senturk, L. M., et al. (1998) Interleukin-8 in the human endometrium. *J Clin Endocrinol Metab*, **83**, 1783-7

Arici, A., Senturk, L. M., Seli, E., et al. (1999) Regulation of monocyte chemotactic protein-1 expression in human endometrial stromal cells by estrogen and progesterone. *Biol Reprod*, **61**, 85-90

Aronica, M. A., Mora, A. L., Mitchell, D. B., et al. (1999) Preferential role for NF-kappa B/Rel signaling in the type 1 but not type 2 T cell-dependent immune response in vivo. *J Immunol*, **163**, 5116-24

Auphan, N., DiDonato, J. A., Rosette, C., et al. (1995) Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. *Science*, **270**, 286-90

Bachelerie, F., Alcami, J., Arenzana-Seisdedos, F., et al. (1991) HIV enhancer activity perpetuated by NF-kappa B induction on infection of monocytes [see comments]. *Nature*, **350**, 709-12

Badinga, L., Michel, F. J., Fields, M. J., et al. (1994) Pregnancy-associated endometrial expression of antileukoproteinase gene is correlated with epitheliochorial placentation. *Mol Reprod Dev*, **38**, 357-363

Badinga, L., Michel, F. J. and Simmen, R. C. (1999) Uterine-associated serine protease inhibitors stimulate deoxyribonucleic acid synthesis in porcine endometrial glandular epithelial cells of pregnancy. *Biol Reprod*, **61**, 380-7

Baeuerle, P. A. and Baltimore, D. (1988) I kappa B: a specific inhibitor of the NF-kappa B transcription factor. *Science*, **242**, 540-6

Baeuerle, P. A., Lenardo, M., Pierce, J. W., et al. (1988) Phorbol-ester-induced activation of the NF-kappa B transcription factor involves dissociation of an apparently cytoplasmic NF-kappa B/inhibitor complex. *Cold Spring Harb Symp Quant Biol*, **53**, 789-98

Baird, D. T., Cameron, S. T., Critchley, H. O., et al. (1996) Prostaglandins and menstruation. *Eur J Obstet Gynecol Reprod Biol*, **70**, 15-7

Baldwin, A. S., Jr. (1996) The NF-kappa B and I kappa B proteins: new discoveries and insights. *Annu Rev Immunol*, **14**, 649-83

Bebington, C., Bell, S. C., Doherty, F. J., et al. (1999) Localization of ubiquitin and ubiquitin cross-reactive protein in human and baboon endometrium and decidua during the menstrual cycle and early pregnancy. *Biol Reprod*, **60**, 920-8

Beck, C. A., Weigel, N. L., Moyer, M. L., et al. (1993) The progesterone antagonist RU486 acquires agonist activity upon stimulation of cAMP signaling pathways. *Proc Natl Acad Sci U S A*, **90**, 4441-5

Beg, A. A. and Baldwin, A. S., Jr. (1993) The I kappa B proteins: multifunctional regulators of Rel/NF-kappa B transcription factors. *Genes Dev*, **7**, 2064-70

Berberich, I., Shu, G. L. and Clark, E. A. (1994) Cross-linking CD40 on B cells rapidly activates nuclear factor-kappa B. *J Immunol*, **153**, 4357-66

Bhatt, H., Brunet, L. J. and Stewart, C. L. (1991) Uterine expression of leukemia inhibitory factor coincides with the onset of blastocyst implantation. *Proc Natl Acad Sci U S A*, **88**, 11408-12

Bhullar, I. S., Li, Y. S., Miao, H., et al. (1998) Fluid shear stress activation of IkappaB kinase is integrin-dependent. *J Biol Chem*, **273**, 30544-9

Birrer, P. (1995) Proteases and antiproteases in cystic fibrosis: pathogenetic considerations and therapeutic strategies. *Respiration*, **62**, 25-8

Bischoff, S. C., Krieger, M., Brunner, T., et al. (1992) Monocyte chemotactic protein 1 is a potent activator of human basophils. *J Exp Med*, **175**, 1271-5

Bohm, B., Aigner, T., Kinne, R., et al. (1992) The serine-protease inhibitor of cartilage matrix is not a chondrocytic gene product. *Eur J Biochem*, **207**, 773-779

Bond, M., Fabunmi, R. P., Baker, A. H., et al. (1998) Synergistic upregulation of metalloproteinase-9 by growth factors and inflammatory cytokines: an absolute requirement for transcription factor NF-kappa B. *FEBS Lett*, **435**, 29-34

Brenner, R. M., Rudolph, L., Matrisian, L., et al. (1996) Non-human primate models; artificial menstrual cycles, endometrial matrix metalloproteinases and s.c. endometrial grafts. *Hum Reprod*, **11 Suppl 2**, 150-64

Brockman, J. A., Scherer, D. C., McKinsey, T. A., et al. (1995) Coupling of a signal response domain in I kappa B alpha to multiple pathways for NF-kappa B activation. *Mol Cell Biol*, **15**, 2809-18

Browatski, M., Schmidt, J., Kuller, W., et al. (2000) Endothelin-1 induces interleukin-6 release via activation of the transcription factor NFκB in human vascular smooth muscle cells. *Basic Res Cardiol*, **95**, 98-105

Brown, K., Gerstberger, S., Carlson, L., et al. (1995) Control of I kappa B-alpha proteolysis by site-specific, signal-induced phosphorylation. *Science*, **267**, 1485-8

Bruner, K. L., Rodgers, W. H., Gold, L. I., et al. (1995) Transforming growth factor beta mediates the progesterone suppression of an epithelial metalloproteinase by adjacent stroma in the human endometrium. *Proc Natl Acad Sci U S A*, **92**, 7362-6

Buckley, C. H. and Fox, H. (1989) Biopsy Pathology of the Endometrium. In Eds Gottlieb, L. S., Neville, A. M. and Walker, R. Biopsy Pathology Series, Chapman and Hall Medical, London.

Bulmer, J. N., Longfellow, M. and Ritson, A. (1991) Leukocytes and resident blood cells in endometrium. *Ann N Y Acad Sci*, **622**, 57-68

Bulmer, J. N., Lunny, D. P. and Hagin, S. V. (1988) Immunohistochemical characterization of stromal leucocytes in nonpregnant human endometrium. *Am J Reprod Immunol Microbiol*, **17**, 83-90

Burrows, T. D., King, A. and Loke, Y. W. (1993) Expression of integrins by human trophoblast and differential adhesion to laminin or fibronectin. *Hum Reprod*, **8**, 475-84

Bush, K. T., Goldberg, A. L. and Nigam, S. K. (1997) Proteasome inhibition leads to a heat-shock response, induction of endoplasmic reticulum chaperones, and thermotolerance. *J Biol Chem*, **272**, 9086-92

Bush, M. R., Mele, J. M., Couchman, G. M., et al. (1998) Evidence of juxtacrine signaling for transforming growth factor alpha in human endometrium. *Biol Reprod*, **59**, 1522-9

Caldenhoven, E., Liden, J., Wissink, S., et al. (1995) Negative cross-talk between RelA and the glucocorticoid receptor: a possible mechanism for the antiinflammatory action of glucocorticoids. *Mol Endocrinol*, **9**, 401-12

Cameron, I. T. (1992) Prostaglandins and menstruation. In Drife, J. O. and Calder, A. A. (eds). Prostaglandins and the Uterus, Springer-Verlag

Cameron, I. T., Leask, R., Kelly, R. W., et al. (1987) Endometrial prostaglandins in women with abnormal menstrual bleeding. *Prostaglandins Leukot Med*, **29**, 249-57

Cameron, I. T., Plumpton, C., Champeney, R., et al. (1993) Identification of endothelin-1, endothelin-2 and endothelin-3 in human endometrium. *J Reprod Fertil*, **98**, 251-5

Cao, Z., Xiong, J., Takeuchi, M., et al. (1996) TRAF6 is a signal transducer for interleukin-1. *Nature*, **383**, 443-6

Carbone, E., Ruggiero, G., Terrazzano, G., et al. (1997) A new mechanism of NK cell cytotoxicity activation: the CD40-CD40 ligand interaction. *J Exp Med*, **185**, 2053-60

Carr, M. W., Roth, S. J., Luther, E., et al. (1994) Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc Natl Acad Sci U S A*, **91**, 3652-6

Carson-Jurica, M. A., Schrader, W. T. and O'Malley, B. W. (1990) Steroid receptor family: structure and functions. *Endocr Rev*, **11**, 201-20

Casey, M. L., Hemsell, D. L., MacDonald, P. C., et al. (1980) NAD+-dependent 15-hydroxyprostaglandin dehydrogenase activity in human endometrium. *Prostaglandins*, **19**, 115-22

Casslen, B., Rosengren, M. and Ohlsson, K. (1981) Localization and quantitation of a low molecular weight proteinase inhibitor, antileukoprotease, in the human uterus. *Hoppe Seylers Z Physiol Chem*, **362**, 953-961

Casslen, B., Sandberg, T., Gustavsson, B., et al. (1998) Transforming growth factor beta1 in the human endometrium. Cyclic variation, increased expression by estradiol and progesterone, and regulation of plasminogen activators and plasminogen activator inhibitor-1. *Biol Reprod*, **58**, 1343-50

Caux, C., Massacrier, C., Vanbervliet, B., et al. (1994) Activation of human dendritic cells through CD40 cross-linking. *J Exp Med*, **180**, 1263-72

Chao, C. C., Lokensgard, J. R., Sheng, W. S., et al. (1997) IL-1-induced iNOS expression in human astrocytes via NF-kappa B. *Neuroreport*, **8**, 3163-6

Charnock-Jones, D. S., Sharkey, A. M., Fenwick, P., et al. (1994) Leukaemia inhibitory factor mRNA concentration peaks in human endometrium at the time of implantation and the blastocyst contains mRNA for the receptor at this time. *J Reprod Fertil*, **101**, 421-6

Chegini, N., Zhao, Y., Williams, R. S., et al. (1994) Human uterine tissue throughout the menstrual cycle expresses transforming growth factor-beta 1 (TGF beta 1), TGF beta 2, TGF beta 3, and TGF beta type II receptor messenger ribonucleic acid and protein and contains [125I]TGF beta 1-binding sites. *Endocrinology*, **135**, 439-49

Chen, D. and Rothenberg, E. V. (1994) Interleukin 2 transcription factors as molecular targets of cAMP inhibition: delayed inhibition kinetics and combinatorial transcription roles. *J Exp Med*, **179**, 931-42

Chen, Z. J., Parent, L. and Maniatis, T. (1996) Site-specific phosphorylation of IkappaBalpha by a novel ubiquitination- dependent protein kinase activity. *Cell*, **84**, 853-62

Cheng, L., Kelly, R. W., Thong, K. J., et al. (1993a) The effect of mifepristone (RU486) on the immunohistochemical distribution of prostaglandin E and its metabolite in decidual and chorionic tissue in early pregnancy. *J Clin Endocrinol Metab*, **77**, 873-7

Cheng, L., Kelly, R. W., Thong, K. J., et al. (1993b) The effects of mifepristone (RU486) on prostaglandin dehydrogenase in decidual and chorionic tissue in early pregnancy. *Hum Reprod*, **8**, 705-9

Cohen, L., Henzel, W. J. and Baeuerle, P. A. (1998) IKAP is a scaffold protein of the IkappaB kinase complex [see comments]. *Nature*, **395**, 292-6

Colditz, I. G. (1990) Effect of exogenous prostaglandin E2 and actinomycin D on plasma leakage induced by neutrophil-activating peptide-1/interleukin-8. *Immunol Cell Biol*, **68**, 397-403

Collett, G. P., Kohnen, G., Campbell, S., et al. (1996) Localization of endothelin receptors in human uterus throughout the menstrual cycle. *Mol Hum Reprod*, **2**, 439-44

Critchley, H. O. D., Bailey, D. A., Au, C. L., et al. (1993) Immunohistochemical sex steroid receptor distribution in endometrium from long-term subdermal levonorgestrel users and during the normal menstrual cycle. *Hum Reprod*, **8**, 1632-9

Critchley, H. O. D., Drudy, T. A., Millar, M. R., et al. (2000) Is Estrogen Receptor Beta (ERβ) Responsible for Steroid Mediated Effects on the Endometrial Vasculature? *J Soc Gynecol Investig*, **7**, 633

Critchley, H. O. D., Jones, R. L., Lea, R. G., et al. (1999) Role of inflammatory mediators in human endometrium during progesterone withdrawal and early pregnancy. *J Clin Endocrinol Metab*, **84**, 240-8

Critchley, H. O. D., Kelly, R. W. and Kooy, J. (1994) Perivascular location of a chemokine interleukin-8 in human endometrium: a preliminary report. *Hum Reprod*, **9**, 1406-9

Critchley, H. O. D., Wang, H., Jones, R. L., et al. (1998a) Morphological and functional features of endometrial decidualization following long-term intrauterine levonorgestrel delivery. *Hum Reprod*, **13**, 1218-24

Critchley, H. O. D., Wang, H., Kelly, R. W., et al. (1998b) Progestin receptor isoforms and prostaglandin dehydrogenase in the endometrium of women using a levonorgestrel-releasing intrauterine system. *Hum Reprod*, **13**, 1210-7

Cushing, S. D., Berliner, J. A., Valente, A. J., et al. (1990) Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc Natl Acad Sci U S A*, **87**, 5134-8

Dalchau, R., Daar, A. S. and Fabre, J. W. (1989) The human Thy-1 molecule. *Immunol Ser*, **45**, 185-96

Damsky, C. H., Fitzgerald, M. L. and Fisher, S. J. (1992) Distribution patterns of extracellular matrix components and adhesion receptors are intricately modulated during first trimester cytotrophoblast differentiation along the invasive pathway, in vivo. *J Clin Invest*, **89**, 210-22

Darby, I., Skalli, O. and Gabbiani, G. (1990) Alpha-smooth muscle actin is transiently expressed by myofibroblasts during experimental wound healing. *Lab Invest*, **63**, 21-9

Delhase, M., Hayakawa, M., Chen, Y., et al. (1999) Positive and negative regulation of IkappaB kinase activity through IKKbeta subunit phosphorylation. *Science*, **284**, 309-13

Denison, F. C., Calder, A. A. and Kelly, R. W. (1999a) The action of prostaglandin E2 on the human cervix: stimulation of interleukin 8 and inhibition of secretory leukocyte protease inhibitor. *Am J Obstet Gynecol*, **180**, 614-20

Denison, F. C., Kelly, R. W. and Calder, A. A. (1997) Differential secretion of chemokines from peripheral blood in pregnant compared with non-pregnant women. *J Reprod Immunol*, **34**, 225-40

Denison, F. C., Kelly, R. W., Calder, A. A., et al. (1999b) Secretory leukocyte protease inhibitor concentration increases in amniotic fluid with the onset of labour in women: characterization of sites of release within the uterus. *J Endocrinol*, **161**, 299-306

DiDonato, J. A., Hayakawa, M., Rothwarf, D. M., et al. (1997) A cytokine-responsive IkappaB kinase that activates the transcription factor NF-kappaB. *Nature*, **388**, 548-54

Draper, D., Donohoe, W., Mortimer, L., et al. (1998) Cysteine proteases of Trichomonas vaginalis degrade secretory leukocyte protease inhibitor. *J Infect Dis*, **178**, 815-819

Economos, K., MacDonald, P. C. and Casey, M. L. (1992) Endothelin-1 gene expression and protein biosynthesis in human endometrium: potential modulator of endometrial blood flow [see comments]. *J Clin Endocrinol Metab*, **74**, 14-9

Edwards, R. G. (1995) Physiological and molecular aspects of human implantation. *Hum Reprod*, **10 Suppl 2**, 1-13

Eigler, A., Siegmund, B., Emmerich, U., et al. (1998) Anti-inflammatory activities of cAMP-elevating agents: enhancement of IL-10 synthesis and concurrent suppression of TNF production. *J Leukoc Biol*, **63**, 101-7

Eisenberg, S. P., Hale, K. K., Heimdal, P., et al. (1990) Location of the protease-inhibitory region of secretory leukocyte protease inhibitor. *J Biol Chem*, **265**, 7976-81

Elner, V. M., Strieter, R. M., Elner, S. G., et al. (1990) Neutrophil chemotactic factor (IL-8) gene expression by cytokine- treated retinal pigment epithelial cells. *Am J Pathol*, **136**, 745-50

Faris, M., Gaskin, F., Parsons, J. T., et al. (1994) CD40 signaling pathway: anti-CD40 monoclonal antibody induces rapid dephosphorylation and phosphorylation of tyrosine-phosphorylated proteins including protein tyrosine kinase Lyn, Fyn, and Syk and the appearance of a 28-kD tyrosine phosphorylated protein. *J Exp Med*, **179**, 1923-31

Finn, C. A. (1986) Implantation, menstruation and inflammation. *Biol Rev Camb Philos Soc*, **61**, 313-28

Finn, C. A. (1987) Why do women and some other primates menstruate? *Perspect Biol Med*, **30**, 566-74

Finn, C. A. (1996) Why do women menstruate? Historical and evolutionary review. *Eur J Obstet Gynecol Reprod Biol*, **70**, 3-8

Finn, C. A. and Pope, M. (1984) Vascular and cellular changes in the decidualized endometrium of the ovariectomized mouse following cessation of hormone treatment: a possible model for menstruation. *J Endocrinol*, **100**, 295-300

Franken, C., Meijer, C. J. and Dijkman, J. H. (1989) Tissue distribution of antileukoprotease and lysozyme in humans. *J Histochem Cytochem*, **37**, 493-498

Fraser, I. S. (1992) Prostaglandins, prostaglandin inhibitors and their roles in gynaecological disorders. *Baillieres Clin Obstet Gynaecol*, **6**, 829-57

Frater-Schroder, M., Risau, W., Hallmann, R., et al. (1987) Tumor necrosis factor type alpha, a potent inhibitor of endothelial cell growth in vitro, is angiogenic in vivo. *Proc Natl Acad Sci U S A*, **84**, 5277-81

Freudenthal, P. S. and Steinman, R. M. (1990) The distinct surface of human blood dendritic cells, as observed after an improved isolation method. *Proc Natl Acad Sci U S A*, **87**, 7698-702

Fries, K. M., Sempowski, G. D., Gaspari, A. A., et al. (1995) CD40 expression by human fibroblasts. *Clin Immunol Immunopathol*, **77**, 42-51

Gallois, C., Habib, A., Tao, J., et al. (1998) Role of NF-kappaB in the antiproliferative effect of endothelin-1 and tumor necrosis factor-alpha in human hepatic stellate cells. Involvement of cyclooxygenase-2. *J Biol Chem*, **273**, 23183-90

Galy, A. H. and Spits, H. (1992) CD40 is functionally expressed on human thymic epithelial cells. *J Immunol*, **149**, 775-82

Gauchat, J. F., Henchoz, S., Fattah, D., et al. (1995) CD40 ligand is functionally expressed on human eosinophils. *Eur J Immunol*, **25**, 863-5

Gauchat, J. F., Henchoz, S., Mazzei, G., et al. (1993) Induction of human IgE synthesis in B cells by mast cells and basophils. *Nature*, **365**, 340-3

Gibb, W., Sun, M. and Kontogiannis, H. (2000) Localization of NFκB in Human Fetal Membranes and Decidua throughout Gestation. *J Soc Gynecol Investig*, **7**, 360

Giudice, L. C. (1999) Potential biochemical markers of uterine receptivity. *Hum Reprod*, **14 Suppl 2**, 3-16

Giudice, L. C., Dsupin, B. A., Jin, I. H., et al. (1993) Differential expression of messenger ribonucleic acids encoding insulin- like growth factors and their receptors in human uterine endometrium and decidua. *J Clin Endocrinol Metab*, **76**, 1115-22

Graf, D., Korthauer, U., Mages, H. W., et al. (1992) Cloning of TRAP, a ligand for CD40 on human T cells. *Eur J Immunol*, **22**, 3191-4

Green, S., Walter, P., Greene, G., et al. (1986) Cloning of the human oestrogen receptor cDNA. *J Steroid Biochem*, **24**, 77-83

Greenland, K. J., Jantke, I., Jenatschke, S., et al. (2000) The human NAD+-dependent 15-hydroxyprostaglandin dehydrogenase gene promoter is controlled by Ets and activating protein-1 transcription factors and progesterone. *Endocrinology*, **141**, 581-97

Gregory, H., Young, J., Schroder, J. M., et al. (1988) Structure determination of a human lymphocyte derived neutrophil activating peptide (LYNAP). *Biochem Biophys Res Commun*, **151**, 883-90

Grove, M. and Plumb, M. (1993) C/EBP, NF-kappa B, and c-Ets family members and transcriptional regulation of the cell-specific and inducible macrophage inflammatory protein 1 alpha immediate-early gene. *Mol Cell Biol*, **13**, 5276-89

Gruss, H. J., Brach, M. A. and Herrmann, F. (1992) Involvement of nuclear factor-kappa B in induction of the interleukin-6 gene by leukemia inhibitory factor. *Blood*, **80**, 2563-70

Haining, R. E., Cameron, I. T., van Papendorp, C., et al. (1991) Epidermal growth factor in human endometrium: proliferative effects in culture and immunocytochemical localization in normal and endometriotic tissues. *Hum Reprod*, **6**, 1200-5

Hampton, A. L. and Salamonsen, L. A. (1994) Expression of messenger ribonucleic acid encoding matrix metalloproteinases and their tissue inhibitors is related to menstruation. *J Endocrinol*, **141**, R1-3

Hanissian, S. H. and Geha, R. S. (1997) Jak3 is associated with CD40 and is critical for CD40 induction of gene expression in B cells. *Immunity*, **6**, 379-87

Harper, M. J. (1992) The implantation window. *Baillieres Clin Obstet Gynaecol*, **6**, 351-71

Hatada, E. N., Krappmann, D. and Scheidereit, C. (2000) NF-kappaB and the innate immune response. *Curr Opin Immunol*, **12**, 52-8

Henn, V., Slupsky, J. R., Grafe, M., et al. (1998) CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature*, **391**, 591-4

Hiemstra, P. S., Maassen, R. J., Stolk, J., et al. (1996) Antibacterial activity of antileukoprotease. *Infect Immun*, **64**, 4520-4524

Higuchi, T., Kanzaki, H., Nakayama, H., et al. (1995) Induction of tissue inhibitor of metalloproteinase 3 gene expression during in vitro decidualization of human endometrial stromal cells. *Endocrinology*, **136**, 4973-81

Hiscott, J., Marois, J., Garoufalis, J., et al. (1993) Characterization of a functional NF-kappa B site in the human interleukin 1 beta promoter: evidence for a positive autoregulatory loop. *Mol Cell Biol*, **13**, 6231-40

Hofmann, G. E., Scott, R. T., Jr., Bergh, P. A., et al. (1991) Immunohistochemical localization of epidermal growth factor in human endometrium, decidua, and placenta. *J Clin Endocrinol Metab*, **73**, 882-7

Holland, P. M., Abramson, R. D., Watson, R., et al. (1991) Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of Thermus aquaticus DNA polymerase. *Proc Natl Acad Sci U S A*, **88**, 7276-80

Hollenbaugh, D., Mischel-Petty, N., Edwards, C. P., et al. (1995) Expression of functional CD40 by vascular endothelial cells. *J Exp Med*, **182**, 33-40

Hornung, D., Ryan, I. P., Chao, V. A., et al. (1997) Immunolocalization and regulation of the chemokine RANTES in human endometrial and endometriosis tissues and cells. *J Clin Endocrinol Metab*, **82**, 1621-8

Horwitz, K. B. and Alexander, P. S. (1983) In situ photolinked nuclear progesterone receptors of human breast cancer cells: subunit molecular weights after transformation and translocation. *Endocrinology*, **113**, 2195-201

Horwitz, K. B., Mockus, M. B. and Lessey, B. A. (1982) Variant T47D human breast cancer cells with high progesterone-receptor levels despite estrogen and antiestrogen resistance. *Cell*, **28**, 633-42

Hsu, H., Huang, J., Shu, H. B., et al. (1996) TNF-dependent recruitment of the protein kinase RIP to the TNF receptor- 1 signaling complex. *Immunity*, **4**, 387-96

Hsu, H., Xiong, J. and Goeddel, D. V. (1995) The TNF receptor 1-associated protein TRADD signals cell death and NF- kappa B activation. *Cell*, **81**, 495-504

Hu, H. M., O'Rourke, K., Boguski, M. S., et al. (1994) A novel RING finger protein interacts with the cytoplasmic domain of CD40. *J Biol Chem*, **269**, 30069-72

Hu, Y., Baud, V., Delhase, M., et al. (1999) Abnormal morphogenesis but intact IKK activation in mice lacking the IKKalpha subunit of IkappaB kinase. *Science*, **284**, 316-20

Hunt, J. S. (1993) Expression and regulation of the tumour necrosis factor-alpha gene in the female reproductive tract. *Reprod Fertil Dev*, **5**, 141-53

Hunt, J. S., Chen, H. L., Hu, X. L., et al. (1992) Tumor necrosis factor-alpha messenger ribonucleic acid and protein in human endometrium. *Biol Reprod*, **47**, 141-7

Huxford, T., Huang, D. B., Malek, S., et al. (1998) The crystal structure of the IkappaBalpha/NF-kappaB complex reveals mechanisms of NF-kappaB inactivation. *Cell*, **95**, 759-70

Iademarco, M. F., McQuillan, J. J., Rosen, G. D., et al. (1992) Characterization of the promoter for vascular cell adhesion molecule-1 (VCAM-1). *J Biol Chem*, **267**, 16323-9

Ibelgaufts, H. (1994) Dictionary of Cytokines. VCH, Weinheim.

Ishida, T., Mizushima, S., Azuma, S., et al. (1996a) Identification of TRAF6, a novel tumor necrosis factor receptor- associated factor protein that mediates signaling from an amino- terminal domain of the CD40 cytoplasmic region. *J Biol Chem*, **271**, 28745-8

Ishida, T. K., Tojo, T., Aoki, T., et al. (1996b) TRAF5, a novel tumor necrosis factor receptor-associated factor family protein, mediates CD40 signaling. *Proc Natl Acad Sci U S A*, **93**, 9437-42

Israel, A. (2000) The IKK complex: an integrator of all signals that activate NF-kappaB? *Trends Cell Biol*, **10**, 129-33

Jacobs, M. D. and Harrison, S. C. (1998) Structure of an IkappaBalpha/NF-kappaB complex. *Cell*, **95**, 749-58

Jeziorska, M., Salamonsen, L. A. and Woolley, D. E. (1995) Mast cell and eosinophil distribution and activation in human endometrium throughout the menstrual cycle. *Biol Reprod*, **53**, 312-20

Jin, F., Nathan, C. F., Radzioch, D., et al. (1998) Lipopolysaccharide-related stimuli induce expression of the secretory leukocyte protease inhibitor, a macrophage-derived lipopolysaccharide inhibitor. *Infect Immun*, **66**, 2447-52

Jin, F. Y., Nathan, C., Radzioch, D., et al. (1997) Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide. *Cell*, **88**, 417-426

Jobin, C. and Sartor, R. B. (2000) The IkappaB/NF-kappaB system: a key determinant of mucosal inflammation and protection. *Am J Physiol Cell Physiol*, **278**, C451-62

Johannisson, E., Parker, R. A., Landgren, B. M., et al. (1982) Morphometric analysis of the human endometrium in relation to peripheral hormone levels. *Fertil Steril*, **38**, 564-71

Johnson, M. H. and Everitt, B. J. (2000) Essential Reproduction. Blackwell Science Ltd.

Jokhi, P. P., King, A. and Loke, Y. W. (1994) Production of granulocyte-macrophage colony-stimulating factor by human trophoblast cells and by decidual large granular lymphocytes. *Hum Reprod*, **9**, 1660-9

Jones, R. L., Kelly, R. W. and Critchley, H. O. D. (1997) Chemokine and cyclooxygenase-2 expression in human endometrium coincides with leukocyte accumulation. *Hum Reprod*, **12**, 1300-6

Junqueira, L. C., Zugaib, M., Montes, G. S., et al. (1980) Morphologic and histochemical evidence for the occurrence of collagenolysis and for the role of neutrophilic polymorphonuclear leukocytes during cervical dilation. *Am J Obstet Gynecol*, **138**, 273-81

Kalkhoven, E., Wissink, S., van der Saag, P. T., et al. (1996) Negative interaction between the RelA(p65) subunit of NF-kappaB and the progesterone receptor. *J Biol Chem*, **271**, 6217-24

Kamat, B. R. and Isaacson, P. G. (1987) The immunocytochemical distribution of leukocytic subpopulations in human endometrium. *Am J Pathol*, **127**, 66-73

Kammerer, U., Marzusch, K., Krober, S., et al. (1999) A subset of CD56+ large granular lymphocytes in first-trimester human decidua are proliferating cells. *Fertil Steril*, **71**, 74-9

Karmann, K., Hughes, C. C., Schechner, J., et al. (1995) CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. *Proc Natl Acad Sci U S A*, **92**, 4342-6

Kastner, P., Krust, A., Turcotte, B., et al. (1990) Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *Embo J*, **9**, 1603-14

Kelly, R. W. (1994) Pregnancy maintenance and parturition: the role of prostaglandin in manipulating the immune and inflammatory response. *Endocr Rev*, **15**, 684-706

Kelly, R. W., Carr, G. G. and Riley, S. C. (1997) The inhibition of synthesis of a beta-chemokine, monocyte chemotactic protein-1 (MCP-1) by progesterone. *Biochem Biophys Res Commun*, **239**, 557-61

Kelly, R. W., Illingworth, P., Baldie, G., et al. (1994) Progesterone control of interleukin-8 production in endometrium and chorio-decidual cells underlines the role of the neutrophil in menstruation and parturition. *Hum Reprod*, **9**, 253-8

Kelly, R. W., Leask, R. and Calder, A. A. (1992) Choriodecidual production of interleukin-8 and mechanism of parturition. *Lancet*, **339**, 776-7

Kiener, P. A., Moran-Davis, P., Rankin, B. M., et al. (1995) Stimulation of CD40 with purified soluble gp39 induces proinflammatory responses in human monocytes. *J Immunol*, **155**, 4917-25

King, A. (2000) Uterine leukocytes and decidualization. *Hum Reprod Update*, **6**, 28-36

King, A., Burrows, T., Verma, S., et al. (1998) Human uterine lymphocytes. *Hum Reprod Update*, **4**, 480-5

King, A., Gardner, L. and Loke, Y. W. (1996) Evaluation of oestrogen and progesterone receptor expression in uterine mucosal lymphocytes. *Hum Reprod*, **11**, 1079-82

King, A. E., Critchley, H. O. and Kelly, R. W. (2000a) Presence of secretory leukocyte protease inhibitor in human endometrium and first trimester decidua suggests an antibacterial protective role. *Mol Hum Reprod*, **6**, 191-6

King, A. E., Kelly, R. W., Critchley, H. O. D., et al. (2000b) CD40 Expression in Uterine Tissues: A Key Regulator of Cytokine Expression by Fibroblasts. *J Clin Endocrinol Metab*, **In press.**

Knudsen, U. B., Uldbjerg, N., Rechberger, T., et al. (1997) Eosinophils in human cervical ripening. *Eur J Obstet Gynecol Reprod Biol*, **72**, 165-8

Koch, A. E., Polverini, P. J., Kunkel, S. L., et al. (1992) Interleukin-8 as a macrophage-derived mediator of angiogenesis [see comments]. *Science*, **258**, 1798-801

Kohnen, G., Campbell, S., Irvine, G. A., et al. (1998) Endothelin receptor expression in human decidua. *Mol Hum Reprod*, **4**, 185-193

Kramps, J. A., van Twisk, C., Appelhans, H., et al. (1990) Proteinase inhibitory activities of antileukoprotease are represented by its second COOH-terminal domain. *Biochim Biophys Acta*, **1038**, 178-85

Kristiansen, F. V., Oster, S., Frost, L., et al. (1987) Isolation of Gardnerella vaginalis in pure culture from the uterine cavity of patients with irregular bleedings. *Br J Obstet Gynaecol*, **94**, 979-984

Kubota, T., Taguchi, M., Kamada, S., et al. (1995) Endothelin synthesis and receptors in human endometrium throughout the normal menstrual cycle. *Hum Reprod*, **10**, 2204-8

Kuiper, G. G., Enmark, E., Pelto-Huikko, M., et al. (1996) Cloning of a novel receptor expressed in rat prostate and ovary. *Proc Natl Acad Sci U S A*, **93**, 5925-30

Kuiper, G. G. and Gustafsson, J. A. (1997) The novel estrogen receptor-beta subtype: potential role in the cell- and promoter-specific actions of estrogens and anti-estrogens. *FEBS Lett*, **410**, 87-90

Laham, N., Rice, G. E., Bishop, G. J., et al. (1993) Interleukin 8 concentrations in amniotic fluid and peripheral venous plasma during human pregnancy and parturition. *Acta Endocrinol (Copenh)*, **129**, 220-4

Laird, S. M., Tuckerman, E., Li, T. C., et al. (1994) Stimulation of human endometrial epithelial cell interleukin 6 production by interleukin 1 and placental protein 14. *Hum Reprod*, **9**, 1339-43

Laird, S. M., Tuckerman, E. M., Cork, B. A., et al. (2000) Expression of nuclear factor kappa B in human endometrium; role in the control of interleukin 6 and leukaemia inhibitory factor production. *Mol Hum Reprod*, **6**, 34-40

Laird, S. M., Tuckerman, E. M., Saravelos, H., et al. (1996) The production of tumour necrosis factor alpha (TNF-alpha) by human endometrial cells in culture. *Hum Reprod*, **11**, 1318-23

Larsen, C. G., Anderson, A. O., Appella, E., et al. (1989a) The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. *Science*, **243**, 1464-6

Larsen, C. G., Anderson, A. O., Oppenheim, J. J., et al. (1989b) Production of interleukin-8 by human dermal fibroblasts and keratinocytes in response to interleukin-1 or tumour necrosis factor. *Immunology*, **68**, 31-6

Leavitt, W. W. and Blaha, G. C. (1972) An estrogen-stimulated, progesterone-binding system in the hamster uterus and vagina. *Steroids*, **19**, 263-74

Lee, D. H. and Goldberg, A. L. (1998) Proteasome inhibitors: valuable new tools for cell biologists. *Trends Cell Biol*, **8**, 397-403

Lee, F. S., Hagler, J., Chen, Z. J., et al. (1997) Activation of the IkappaB alpha kinase complex by MEKK1, a kinase of the JNK pathway. *Cell*, **88**, 213-22

Lee, F. S., Peters, R. T., Dang, L. C., et al. (1998) MEKK1 activates both IkappaB kinase alpha and IkappaB kinase beta. *Proc Natl Acad Sci U S A*, **95**, 9319-24

Lentsch, A. B., Jordan, J. A., Czermak, B. J., et al. (1999) Inhibition of NF-kappaB activation and augmentation of IkappaBbeta by secretory leukocyte protease inhibitor during lung inflammation. *Am J Pathol*, **154**, 239-47

Lentsch, A. B., Shanley, T. P., Sarma, V., et al. (1997) In vivo suppression of NF-kappa B and preservation of I kappa B alpha by interleukin-10 and interleukin-13. *J Clin Invest*, **100**, 2443-8

Lessey, B. A., Alexander, P. S. and Horwitz, K. B. (1983) The subunit structure of human breast cancer progesterone receptors: characterization by chromatography and photoaffinity labeling. *Endocrinology*, **112**, 1267-74

Lessey, B. A., Castelbaum, A. J., Buck, C. A., et al. (1994) Further characterization of endometrial integrins during the menstrual cycle and in pregnancy [see comments]. *Fertil Steril*, **62**, 497-506

Lessey, B. A., Castelbaum, A. J., Sawin, S. W., et al. (1995) Integrins as markers of uterine receptivity in women with primary unexplained infertility. *Fertil Steril*, **63**, 535-42

Lessey, B. A., Damjanovich, L., Coutifaris, C., et al. (1992) Integrin adhesion molecules in the human endometrium. Correlation with the normal and abnormal menstrual cycle. *J Clin Invest*, **90**, 188-95

Lessey, B. A., Killam, A. P., Metzger, D. A., et al. (1988) Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. *J Clin Endocrinol Metab*, **67**, 334-40

Li, Q., Van Antwerp, D., Mercurio, F., et al. (1999) Severe liver degeneration in mice lacking the IkappaB kinase 2 gene. *Science*, **284**, 321-5

Li, T. C., Warren, M. A., Hill, C. J., et al (1994) Morphology of the human endometrium in the peri-implantation period. *Ann N Y Acad Sci*, **734**, 169-84

Lin, Y. Z., Yao, S. Y., Veach, R. A., et al. (1995) Inhibition of nuclear translocation of transcription factor NF-kappa B by a synthetic peptide containing a cell membrane-permeable motif and nuclear localization sequence. *J Biol Chem*, **270**, 14255-8

Ling, L., Cao, Z. and Goeddel, D. V. (1998) NF-kappaB-inducing kinase activates IKK-alpha by phosphorylation of Ser- 176. *Proc Natl Acad Sci U S A*, **95**, 3792-7

Liu, L., Paul, A. and Plevin, R. (1999) The effect of NF-kappa B inhibitors on IRF-1 expression in RAW 264.7 macrophages. *British J Pharmacol*, **128**, 146P

Lockwood, C. J., Krikun, G., Hausknecht, V. A., et al. (1998) Matrix metalloproteinase and matrix metalloproteinase inhibitor expression in endometrial

stromal cells during progestin-initiated decidualization and menstruation-related progestin withdrawal. *Endocrinology*, **139**, 4607-13

Loke, Y. W. and King, A. (1995) Human Implantation, Cell Biology and Immunology. In Eds Loke, Y. W. and King, A. Cambridge University Press, Cambridge.

Loke, Y. W. and King, A. (1997) Immunology of human placental implantation: clinical implications of our current understanding. *Mol Med Today*, **3**, 153-9

Ludwig, H. and Spornitz, U. M. (1991) Microarchitecture of the human endometrium by scanning electron microscopy: menstrual desquamation and remodeling. *Ann N Y Acad Sci*, **622**, 28-46

Lumsden, M. A., Brown, A. and Baird, D. T. (1984) Prostaglandin production from homogenates of separated glandular epithelium and stroma from human endometrium. *Prostaglandins*, **28**, 485-96

Luster, A. D. (1998) Chemokines--chemotactic cytokines that mediate inflammation. *N Engl J Med*, **338**, 436-45

Mach, F., Schonbeck, U., Sukhova, G. K., et al. (1997) Functional CD40 ligand is expressed on human vascular endothelial cells, smooth muscle cells, and macrophages: implications for CD40-CD40 ligand signaling in atherosclerosis. *Proc Natl Acad Sci U S A*, **94**, 1931-6

Malinin, N. L., Boldin, M. P., Kovalenko, A. V., et al. (1997) MAP3K-related kinase involved in NF-kappaB induction by TNF, CD95 and IL-1. *Nature*, **385**, 540-4

Manna, S. K., Mukhopadhyay, A. and Aggarwal, B. B. (2000) Human chorionic gonadotropin suppresses activation of nuclear transcription factor-kappa B and activator protein-1 induced by tumor necrosis factor. *J Biol Chem*, **275**, 13307-14

Manni, A., Kleimberg, J., Ackerman, V., et al. (1996) Inducibility of RANTES mRNA by IL-1beta in human bronchial epithelial cells is associated with increased NF-kappaB DNA binding activity. *Biochem Biophys Res Commun*, **220**, 120-4

Marions, L. and Danielsson, K. G. (1999) Expression of cyclo-oxygenase in human endometrium during the implantation period. *Mol Hum Reprod*, **5**, 961-5

Markee, J. E. (1940) Menstruation in intraocular endometrial transplants in the rhesus monkey. *Contrib Embryol Carnegie Inst*, **177**, 211-308

Marsh, M. M., Findlay, J. K. and Salamonsen, L. A. (1996) Endothelin and menstruation. *Hum Reprod*, **11 Suppl 2**, 83-9

Marshall, J. C. and Odell, W. D. (1989) The Menstrual Cycle - Hormonal Regulation, Mechanisms of Anovulation and Responses of the Reproductive Tract to Steroid Hormones. In L. J. DeGroot (eds), *Endocrinology*. W B Saunders Co,1940-1949.

Martel, D., Malet, C., Gautray, J. P., et al. (1989) Surface changes of the luminal uterine epithelium during the human menstrual cycle: a scanning electron microscope study. In J. de Brux, R. Mortel and J. P. Guatray (eds), *The Endometrium: Hormonal Impacts*. Plenum Press,New York.

Martin, T., Cardarelli, P. M., Parry, G. C., et al. (1997) Cytokine induction of monocyte chemoattractant protein-1 gene expression in human endothelial cells depends on the cooperative action of NF-kappa B and AP-1. *Eur J Immunol*, **27**, 1091-7

Maruyama, M., Hay, J. G., Yoshimura, K., et al. (1994) Modulation of secretory leukoprotease inhibitor gene expression in human bronchial epithelial cells by phorbol ester. *J Clin Invest*, **94**, 368-75

Marzusch, K., Ruck, P., Geiselhart, A., et al. (1993) Distribution of cell adhesion molecules on CD56++, CD3-, CD16- large granular lymphocytes and endothelial cells in first-trimester human decidua. *Hum Reprod*, **8**, 1203-8

Matsukura, S., Stellato, C., Plitt, J. R., et al. (1999) Activation of eotaxin gene transcription by NF-kappa B and STAT6 in human airway epithelial cells. *J Immunol*, **163**, 6876-83

Matsuzaki, S., Fukaya, T., Suzuki, T., et al. (1999) Oestrogen receptor alpha and beta mRNA expression in human endometrium throughout the menstrual cycle. *Mol Hum Reprod*, **5**, 559-64

Mauviel, A., Reitamo, S., Remitz, A., et al. (1992) Leukoregulin, a T cell-derived cytokine, induces IL-8 gene expression and secretion in human skin fibroblasts. Demonstration and secretion in human skin fibroblasts. Demonstration of enhanced NF-kappa B binding and NF-kappa B-driven promoter activity. *J Immunol*, **149**, 2969-76

May, M. J. and Ghosh, S. (1999) IkappaB kinases: kinsmen with different crafts. *Science*, **284**, 271-3

McDonnell, D. P. and Goldman, M. E. (1994) RU486 exerts antiestrogenic activities through a novel progesterone receptor A form-mediated mechanism. *J Biol Chem*, **269**, 11945-9

McDonnell, D. P., Shahbaz, M. M., Vegeto, E., et al. (1994) The human progesterone receptor A-form functions as a transcriptional modulator of mineralocorticoid receptor transcriptional activity. *J Steroid Biochem Mol Biol*, **48**, 425-32

McKay, L. I. and Cidlowski, J. A. (1998) Cross-talk between nuclear factor-kappa B and the steroid hormone receptors: mechanisms of mutual antagonism. *Mol Endocrinol*, **12**, 45-56

McKay, L. I. and Cidlowski, J. A. (1999) Molecular control of immune/inflammatory responses: interactions between nuclear factor-kappa B and steroid receptor-signaling pathways. *Endocr Rev*, **20**, 435-59

McNeely, T. B., Dealy, M., Dripps, D. J., et al. (1995) Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. *J Clin Invest*, **96**, 456-64

McNeely, T. B., Shugars, D. C., Rosendahl, M., et al. (1997) Inhibition of human immunodeficiency virus type 1 infectivity by secretory leukocyte protease inhibitor occurs prior to viral reverse transcription. *Blood*, **90**, 1141-1149

Mercurio, F. and Manning, A. M. (1999) Multiple signals converging on NF-kappaB. *Curr Opin Cell Biol*, **11**, 226-32

Mercurio, F., Murray, B. W., Shevchenko, A., et al. (1999) IkappaB kinase (IKK)-associated protein 1, a common component of the heterogeneous IKK complex. *Mol Cell Biol*, **19**, 1526-38

Mercurio, F., Zhu, H., Murray, B. W., et al. (1997) IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF- kappaB activation. *Science*, **278**, 860-6

Milgrom, E., Atger, M. and Baulieu, E. E. (1970) Progesterone in uterus and plasma. IV. Progesterone receptor(s) in guinea pig uterus cytosol. *Steroids*, **16**, 741-54

Miller, K. W., Evans, R. J., Eisenberg, S. P., et al. (1989) Secretory leukocyte protease inhibitor binding to mRNA and DNA as a possible cause of toxicity to Escherichia coli. *J Bacteriol*, **171**, 2166-72

Miller, L. and Hunt, J. S. (1998) Regulation of TNF-alpha production in activated mouse macrophages by progesterone. *J Immunol*, **160**, 5098-104

Milne, S. A., Critchley, H. O., Drudy, T. A., et al. (1999) Perivascular interleukin-8 messenger ribonucleic acid expression in human endometrium varies across the menstrual cycle and in early pregnancy decidua. *J Clin Endocrinol Metab*, **84**, 2563-7

Ming, W. J., Bersani, L. and Mantovani, A. (1987) Tumor necrosis factor is chemotactic for monocytes and polymorphonuclear leukocytes. *J Immunol*, **138**, 1469-74

Moller, B. R., Kristiansen, F. V., Thorsen, P., et al. (1995) Sterility of the uterine cavity. *Acta Obstet Gynecol Scand*, **74**, 216-219

Moore, K. W., O'Garra, A., de Waal Malefyt, R., et al. (1993) Interleukin-10. *Annu Rev Immunol*, **11**, 165-90

Mosselman, S., Polman, J. and Dijkema, R. (1996) ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett*, **392**, 49-53

Mote, P. A., Balleine, R. L., McGowan, E. M., et al. (1999) Colocalization of progesterone receptors A and B by dual immunofluorescent histochemistry in human endometrium during the menstrual cycle. *J Clin Endocrinol Metab*, **84**, 2963-71

Mukaida, N., Mahe, Y. and Matsushima, K. (1990) Cooperative interaction of nuclear factor-kappa B- and cis-regulatory enhancer binding protein-like factor binding elements in activating the interleukin-8 gene by pro-inflammatory cytokines. *J Biol Chem*, **265**, 21128-33

Muroi, M. and Suzuki, T. (1993) Role of protein kinase A in LPS-induced activation of NF-kappa B proteins of a mouse macrophage-like cell line, J774. *Cell Signal*, **5**, 289-98

Murphy, P. M. (1996) Chemokine receptors: structure, function and role in microbial pathogenesis. *Cytokine Growth Factor Rev*, **7**, 47-64

Nachtigall, M. J., Kliman, H. J., Feinberg, R. F., et al. (1996) The effect of leukemia inhibitory factor (LIF) on trophoblast differentiation: a potential role in human implantation. *J Clin Endocrinol Metab*, **81**, 801-6

Nakano, H., Shindo, M., Sakon, S., et al. (1998) Differential regulation of IkappaB kinase alpha and beta by two upstream kinases, NF-kappaB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. *Proc Natl Acad Sci U S A*, **95**, 3537-42

Narumi, S., Tebo, J. M., Finke, J. H., et al. (1992) IFN-gamma and IL-2 cooperatively activate NF kappa B in murine peritoneal macrophages. *J Immunol*, **149**, 529-34

Nemoto, S., DiDonato, J. A. and Lin, A. (1998) Coordinate regulation of IkappaB kinases by mitogen-activated protein kinase kinase kinase 1 and NF-kappaB-inducing kinase. *Mol Cell Biol*, **18**, 7336-43

Neumann, M., Grieshammer, T., Chuvpilo, S., et al. (1995) RelA/p65 is a molecular target for the immunosuppressive action of protein kinase A. *Embo J*, **14**, 1991-2004

Nguyen, H., Teskey, L., Lin, R., et al. (1999) Identification of the secretory leukocyte protease inhibitor (SLPI) as a target of IRF-1 regulation. *Oncogene*, **18**, 5455-63

Ninomiya-Tsuji, J., Kishimoto, K., Hiyama, A., et al. (1999) The kinase TAK1 can activate the NIK-I kappaB as well as the MAP kinase cascade in the IL-1 signalling pathway. *Nature*, **398**, 252-6

Nishida, M., Kasahara, K., Kaneko, M., et al. (1985) [Establishment of a new human endometrial adenocarcinoma cell line, Ishikawa cells, containing estrogen and progesterone receptors]. *Nippon Sanka Fujinka Gakkai Zasshi*, **37**, 1103-11

Noyes, R. W., Hertig, A. T. and Rock, J. (1950) Dating the Endometrial Biopsy. *Fertility and Sterility*, **1**, 3-25

O'Reilly, G., Charnock-Jones, D. S., Davenport, A. P., et al. (1992) Presence of messenger ribonucleic acid for endothelin-1, endothelin-2, and endothelin-3 in human endometrium and a change in the ratio of ETA and ETB receptor subtype across the menstrual cycle. *J Clin Endocrinol Metab*, **75**, 1545-9

Ohbuchi, H., Nagai, K., Yamaguchi, M., et al. (1995) Endothelin-1 and big endothelin-1 increase in human endometrium during menstruation. *Am J Obstet Gynecol*, **173**, 1483-90

Ohlsson, K., Bjartell, A. and Lilja, H. (1995) Secretory leucocyte protease inhibitor in the male genital tract: PSA- induced proteolytic processing in human semen and tissue localization. *J Androl*, **16**, 64-74

Old, L. J. (1985) Tumor necrosis factor (TNF). *Science*, **230**, 630-2

Oliver, C., Montes, M. J., Galindo, J. A., et al. (1999) Human decidual stromal cells express alpha-smooth muscle actin and show ultrastructural similarities with myofibroblasts. *Hum Reprod*, **14**, 1599-605

Osborn, L., Kunkel, S. and Nabel, G. J. (1989) Tumor necrosis factor alpha and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor kappa B. *Proc Natl Acad Sci U S A*, **86**, 2336-40

Oster, W., Brach, M. A., Gruss, H. J., et al. (1992) Interleukin-1 beta (IL-1 beta) expression in human blood mononuclear phagocytes is differentially regulated by

granulocyte-macrophage colony- stimulating factor (GM-CSF), M-CSF, and IL-3. *Blood*, **79**, 1260-5

Patel, A., Hanson, J., McLean, T. I., et al. (1998) Herpes simplex type 1 induction of persistent NF-kappa B nuclear translocation increases the efficiency of virus replication. *Virology*, **247**, 212-22

Pekonen, F., Nyman, T., Lahteenmaki, P., et al. (1992) Intrauterine progestin induces continuous insulin-like growth factor- binding protein-1 production in the human endometrium. *J Clin Endocrinol Metab*, **75**, 660-4

Perrot-Applanat, M., Deng, M., Fernandez, H., et al. (1994) Immunohistochemical localization of estradiol and progesterone receptors in human uterus throughout pregnancy: expression in endometrial blood vessels. *J Clin Endocrinol Metab*, **78**, 216-24

Philippeaux, M. M. and Piguet, P. F. (1993) Expression of tumor necrosis factor-alpha and its mRNA in the endometrial mucosa during the menstrual cycle. *Am J Pathol*, **143**, 480-6

Pickles, V. R. (1957) A Plain-muscle Stimulant in the Menstruum. *Nature* , **180**, 1198-1199

Pijnenborg, R., Robertson, W. B., Brosens, I., et al. (1981) Review article: trophoblast invasion and the establishment of haemochorial placentation in man and laboratory animals. *Placenta*, **2**, 71-91

Pomerantz, J. L. and Baltimore, D. (1999) NF-kappaB activation by a signaling complex containing TRAF2, TANK and TBK1, a novel IKK-related kinase. *Embo J*, **18**, 6694-704

Poppe, W. A., Drijkoningen, M., Ide, P. S., et al. (1998) Lymphocytes and dendritic cells in the normal uterine cervix. An immunohistochemical study. *Eur J Obstet Gynecol Reprod Biol*, **81**, 277-82

Poropatich, C., Rojas, M. and Silverberg, S. G. (1987) Polymorphonuclear leukocytes in the endometrium during the normal menstrual cycle. *Int J Gynecol Pathol*, **6**, 230-4

Psychoyos, A., Nikas, G. and Gravanis, A. (1995) The role of prostaglandins in blastocyst implantation. *Hum Reprod*, **10 Suppl 2**, 30-42

Quayle, A. J., Porter, E. M., Nussbaum, A. A., et al. (1998) Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. *Am J Pathol*, **152**, 1247-1258

Rampart, M., Van Damme, J., Zonnekeyn, L., et al. (1989) Granulocyte chemotactic protein/interleukin-8 induces plasma leakage and neutrophil accumulation in rabbit skin. *Am J Pathol*, **135**, 21-5

Ray, A. and Prefontaine, K. E. (1994) Physical association and functional antagonism between the p65 subunit of transcription factor NF-kappa B and the glucocorticoid receptor. *Proc Natl Acad Sci U S A*, **91**, 752-6

Reed, K. L., Badinga, L., Davis, D. L., et al. (1996) Porcine endometrial glandular epithelial cells in vitro: transcriptional activities of the pregnancy-associated genes encoding antileukoproteinase and uteroferrin. *Biol Reprod*, **55**, 469-477

Reed, K. L., Blaeser, L. L., Dantzer, V., et al. (1998) Control of secretory leukocyte protease inhibitor gene expression in the porcine periimplantation endometrium: a case of maternal-embryo communication. *Biol Reprod*, **58**, 448-57

Reed, K. L., Michel, F. J., Hiemstra, P. S., et al. (1997) Interaction of Secretory Leukocyte Protease Inhibitor with Placental and Endometrial Extracellular Matrix. *Biol Reprod*, **56**, 489

Rees, M. C., Anderson, A. B., Demers, L. M., et al. (1984) Prostaglandins in menstrual fluid in menorrhagia and dysmenorrhoea. *Br J Obstet Gynaecol*, **91**, 673-80

Regnier, C. H., Song, H. Y., Gao, X., et al. (1997) Identification and characterization of an IkappaB kinase. *Cell*, **90**, 373-83

Ren, C. L., Morio, T., Fu, S. M., et al. (1994) Signal transduction via CD40 involves activation of lyn kinase and phosphatidylinositol-3-kinase, and phosphorylation of phospholipase C gamma 2. *J Exp Med*, **179**, 673-80

Revelli, A., Massobrio, M. and Tesarik, J. (1998) Nongenomic actions of steroid hormones in reproductive tissues. *Endocr Rev*, **19**, 3-17

Richardson, C. A., Couser, W. G., Iwasaki, T., et al. (1991) Lipopolysaccharide (LPS) induced NF-kappa B like DNA-binding activity in glomerular cells is blocked by herbimycin A. *Clinical Research*, **39**, A307

Roberts, D. K., Parmley, T. H., Walker, N. J., et al. (1992) Ultrastructure of the microvasculature in the human endometrium throughout the normal menstrual cycle. *Am J Obstet Gynecol*, **166**, 1393-406

Rodgers, W. H., Matrisian, L. M., Giudice, L. C., et al. (1994) Patterns of matrix metalloproteinase expression in cycling endometrium imply differential functions and regulation by steroid hormones. *J Clin Invest*, **94**, 946-53

Rogers, P. A., Abberton, K. M. and Susil, B. (1992) Endothelial cell migratory signal produced by human endometrium during the menstrual cycle. *Hum Reprod*, **7**, 1061-6

Rollins, B. J. (1991) JE/MCP-1: an early-response gene encodes a monocyte-specific cytokine. *Cancer Cells*, **3**, 517-24

Rollins, B. J. (1997) Chemokines. *Blood*, **90**, 909-28

Romero, R., Avila, C., Santhanam, U., et al. (1990) Amniotic fluid interleukin 6 in preterm labor. Association with infection. *J Clin Invest*, **85**, 1392-400

Romero, R., Ceska, M., Avila, C., et al. (1991) Neutrophil attractant/activating peptide-1/interleukin-8 in term and preterm parturition. *Am J Obstet Gynecol*, **165**, 813-20

Romero, R., Sirtori, M., Oyarzun, E., et al. (1989) Infection and labor. V. Prevalence, microbiology, and clinical significance of intraamniotic infection in women with preterm labor and intact membranes. *Am J Obstet Gynecol*, **161**, 817-24

Rothe, M., Wong, S. C., Henzel, W. J., et al. (1994) A novel family of putative signal transducers associated with the cytoplasmic domain of the 75 kDa tumor necrosis factor receptor. *Cell*, **78**, 681-92

Rothwarf, D. M., Zandi, E., Natoli, G., et al. (1998) IKK-gamma is an essential regulatory subunit of the IkappaB kinase complex. *Nature*, **395**, 297-300

Salamonsen, L. A. (1998) Current concept of the mechanisms of menstruation: A normal process of tissue destruction. *Trends Endocrinol Metab*, **9**, 305-9

Salamonsen, L. A., Butt, A. R., Hammond, F. R., et al. (1997) Production of endometrial matrix metalloproteinases, but not their tissue inhibitors, is modulated by progesterone withdrawal in an in vitro model for menstruation. *J Clin Endocrinol Metab*, **82**, 1409-15

Salamonsen, L. A., Butt, A. R., Macpherson, A. M., et al. (1992) Immunolocalization of the vasoconstrictor endothelin in human endometrium during the menstrual cycle and in umbilical cord at birth. *Am J Obstet Gynecol*, **167**, 163-7

Salamonsen, L. A. and Lathbury, L. J. (2000) Endometrial leukocytes and menstruation. *Hum Reprod Update*, **6**, 16-27

Salamonsen, L. A. and Woolley, D. E. (1996) Matrix metalloproteinases in normal menstruation. *Hum Reprod*, **11 Suppl 2**, 124-33

Salamonsen, L. A. and Woolley, D. E. (1999) Menstruation: induction by matrix metalloproteinases and inflammatory cells. *J Reprod Immunol*, **44**, 1-27

Sallenave, J. M., Shulmann, J., Crossley, J., et al. (1994) Regulation of secretory leukocyte proteinase inhibitor (SLPI) and elastase-specific inhibitor (ESI/elafin) in human airway epithelial cells by cytokines and neutrophilic enzymes. *Am J Respir Cell Mol Biol*, **11**, 733-41

Scheinman, R. I., Cogswell, P. C., Lofquist, A. K., et al. (1995a) Role of transcriptional activation of I kappa B alpha in mediation of immunosuppression by glucocorticoids. *Science*, **270**, 283-6

Scheinman, R. I., Gualberto, A., Jewell, C. M., et al. (1995b) Characterization of mechanisms involved in transrepression of NF-kappa B by activated glucocorticoid receptors. *Mol Cell Biol*, **15**, 943-53

Scherer, D. C., Brockman, J. A., Chen, Z., et al. (1995) Signal-induced degradation of I kappa B alpha requires site-specific ubiquitination. *Proc Natl Acad Sci U S A*, **92**, 11259-63

Schoenberger, S. P., Toes, R. E., van der Voort, E. I., et al. (1998) T-cell help for cytotoxic T lymphocytes is mediated by CD40-CD40L interactions [see comments]. *Nature*, **393**, 480-3

Schottelius, A. J., Mayo, M. W., Sartor, R. B., et al. (1999) Interleukin-10 signaling blocks inhibitor of kappaB kinase activity and nuclear factor kappa B DNA binding. *J Biol Chem*, **274**, 31868-74

Schurch, W., Seemayer, T. A. and Gabbiani, G. (1992) Myofibroblasts. In S. S. Sternberg (eds), *Histology for Pathologists*. Raven Press Ltd,New York,p. 109-144.

Sempowski, G. D., Chess, P. R., Moretti, A. J., et al. (1997a) CD40 mediated activation of gingival and periodontal ligament fibroblasts. *J Periodontol*, **68**, 284-92

Sempowski, G. D., Chess, P. R. and Phipps, R. P. (1997b) CD40 is a functional activation antigen and B7-independent T cell costimulatory molecule on normal human lung fibroblasts. *J Immunol*, **158**, 4670-7

Sempowski, G. D., Rozenblit, J., Smith, T. J., et al. (1998) Human orbital fibroblasts are activated through CD40 to induce proinflammatory cytokine production. *Am J Physiol*, **274**, C707-14

Sen, R. and Baltimore, D. (1986) Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell*, **46**, 705-16

Sennstrom, M. B., Ekman, G., Westergren-Thorsson, G., et al. (2000) Human cervical ripening, an inflammatory process mediated by cytokines. *Mol Hum Reprod*, **6**, 375-81

Sennstrom, M. K., Brauner, A., Lu, Y., et al. (1997) Interleukin-8 is a mediator of the final cervical ripening in humans. *Eur J Obstet Gynecol Reprod Biol*, **74**, 89-92

Serkkola, E. and Hurme, M. (1993) Activation of NF-kappa B by cAMP in human myeloid cells. *FEBS Lett*, **334**, 327-30

Shakhov, A. N., Collart, M. A., Vassalli, P., et al. (1990) Kappa B-type enhancers are involved in lipopolysaccharide-mediated transcriptional activation of the tumor necrosis factor alpha gene in primary macrophages. *J Exp Med*, **171**, 35-47

Sherman, M. R., Corvol, P. L. and O'Malley, B. W. (1970) Progesterone-binding components of chick oviduct. I. Preliminary characterization of cytoplasmic components. *J Biol Chem*, **245**, 6085-96

Sheth, K. V., Roca, G. L., al-Sedairy, S. T., et al. (1991) Prediction of successful embryo implantation by measuring interleukin-1- alpha and immunosuppressive factor(s) in preimplantation embryo culture fluid. *Fertil Steril*, **55**, 952-7

Shimada, T., Kawai, T., Takeda, K., et al. (1999) IKK-i, a novel lipopolysaccharide-inducible kinase that is related to IkappaB kinases. *Int Immunol*, **11**, 1357-62

Shimonovitz, S., Hurwitz, A., Dushnik, M., et al. (1994) Developmental regulation of the expression of 72 and 92 kd type IV collagenases in human trophoblasts: a possible mechanism for control of trophoblast invasion [see comments]. *Am J Obstet Gynecol*, **171**, 832-8

Sica, A., Dorman, L., Viggiano, V., et al. (1997) Interaction of NF-kappaB and NFAT with the interferon-gamma promoter. *J Biol Chem*, **272**, 30412-20

Sica, A., Wang, J. M., Colotta, F., et al. (1990) Monocyte chemotactic and activating factor gene expression induced in endothelial cells by IL-1 and tumor necrosis factor. *J Immunol*, **144**, 3034-8

Siebenlist, U., Franzoso, G. and Brown, K. (1994) Structure, regulation and function of NF-kappa B. *Annu Rev Cell Biol*, **10**, 405-55

Siiteri, P. K. and Stites, D. P. (1982) Immunologic and endocrine interrelationships in pregnancy. *Biol Reprod*, **26**, 1-14

Simon, C., Frances, A., Lee, B. Y., et al. (1995) Immunohistochemical localization, identification and regulation of the interleukin-1 receptor antagonist in the human endometrium. *Hum Reprod*, **10**, 2472-7

Simon, C., Frances, A., Piquette, G., et al. (1994a) Interleukin-1 system in the materno-trophoblast unit in human implantation: immunohistochemical evidence for autocrine/paracrine function. *J Clin Endocrinol Metab*, **78**, 847-54

Simon, C., Frances, A., Piquette, G. N., et al. (1994b) Embryonic implantation in mice is blocked by interleukin-1 receptor antagonist. *Endocrinology*, **134**, 521-8

Simon, C., Gimeno, M. J., Mercader, A., et al. (1996) Cytokines-adhesion molecules-invasive proteinases. The missing paracrine/autocrine link in embryonic implantation? *Mol Hum Reprod*, **2**, 405-24

Simon, C., Piquette, G. N., Frances, A., et al. (1993a) Localization of interleukin-1 type I receptor and interleukin-1 beta in human endometrium throughout the menstrual cycle. *J Clin Endocrinol Metab*, **77**, 549-55

Simon, C., Piquette, G. N., Frances, A., et al. (1993b) Interleukin-1 type I receptor messenger ribonucleic acid expression in human endometrium throughout the menstrual cycle. *Fertil Steril*, **59**, 791-6

Slayden, O. D., Koji, T. and Brenner, R. M. (1995) Microwave stabilization enhances immunocytochemical detection of estrogen receptor in frozen sections of macaque oviduct. *Endocrinology*, **136**, 4012-21

Smith, R. S., Smith, T. J., Blieden, T. M., et al. (1997) Fibroblasts as sentinel cells. Synthesis of chemokines and regulation of inflammation. *Am J Pathol*, **151**, 317-22

Smith, S. K. (1998) Angiogenesis, vascular endothelial growth factor and the endometrium. *Hum Reprod Update*, **4**, 509-19

Smith, T. J., Sempowski, G. D., Wang, H. S., et al. (1995) Evidence for cellular heterogeneity in primary cultures of human orbital fibroblasts. *J Clin Endocrinol Metab*, **80**, 2620-5

Snijders, M., de Goeij, A., Koudstaal, J., et al. (1996) Oestrogen and progestogen receptor content in human endometrium. *Eur J Obstet Gynecol Reprod Biol*, **70**, 9-10

Song, H. Y., Regnier, C. H., Kirschning, C. J., et al. (1997) Tumor necrosis factor (TNF)-mediated kinase cascades: bifurcation of nuclear factor-kappaB and c-jun N-terminal kinase (JNK/SAPK) pathways at TNF receptor-associated factor 2. *Proc Natl Acad Sci U S A*, **94**, 9792-6

Stamenkovic, I., Clark, E. A. and Seed, B. (1989) A B-lymphocyte activation molecule related to the nerve growth factor receptor and induced by cytokines in carcinomas. *Embo J*, **8**, 1403-10

Stancovski, I. and Baltimore, D. (1997) NF-kappaB activation: the I kappaB kinase revealed? *Cell*, **91**, 299-302

Stewart, C. L., Kaspar, P., Brunet, L. J., et al. (1992) Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor [see comments]. *Nature*, **359**, 76-9

Strassmann, G., Patil-Koota, V., Finkelman, F., et al. (1994) Evidence for the involvement of interleukin 10 in the differential deactivation of murine peritoneal macrophages by prostaglandin E2. *J Exp Med*, **180**, 2365-70

Strauss III, J. F. and Gurpide, E. (1991) The Endometrium: Regulation and Dysfunction. In S. S. C. Yen and R. B. Jaffe (eds), *Reproductive Endocrinology - Physiology, Pathophysiology and Clinical Management*. W B Saunders Co,309-338.

Strieter, R. M., Kunkel, S. L., Showell, H. J., et al. (1988) Monokine-induced gene expression of a human endothelial cell-derived neutrophil chemotactic factor. *Biochem Biophys Res Commun*, **156**, 1340-5

Sun, S. C., Ganchi, P. A., Ballard, D. W., et al. (1993) NF-kappa B controls expression of inhibitor I kappa B alpha: evidence for an inducible autoregulatory pathway. *Science*, **259**, 1912-5

Tabibzadeh, S. (1991) Human endometrium: an active site of cytokine production and action. *Endocr Rev*, **12**, 272-90

Tabibzadeh, S. (1992) Patterns of expression of integrin molecules in human endometrium throughout the menstrual cycle. *Hum Reprod*, **7**, 876-82

Tabibzadeh, S. (1994) Regulatory roles of IFN-gamma in human endometrium. *Ann N Y Acad Sci*, **734**, 1-6

Tabibzadeh, S. (1996) The signals and molecular pathways involved in human menstruation, a unique process of tissue destruction and remodelling. *Mol Hum Reprod*, **2**, 77-92

Tabibzadeh, S. and Babaknia, A. (1995) The signals and molecular pathways involved in implantation, a symbiotic interaction between blastocyst and endometrium involving adhesion and tissue invasion. *Hum Reprod*, **10**, 1579-602

Tabibzadeh, S., Kaffka, K. L., Satyaswaroop, P. G., et al. (1990) Interleukin-1 (IL-1) regulation of human endometrial function: presence of IL-1 receptor correlates with IL-1-stimulated prostaglandin E2 production. *J Clin Endocrinol Metab*, **70**, 1000-6

Tabibzadeh, S., Kong, Q. F., Babaknia, A., et al. (1995a) Progressive rise in the expression of interleukin-6 in human endometrium during menstrual cycle is initiated during the implantation window. *Hum Reprod*, **10**, 2793-9

Tabibzadeh, S., Satyaswaroop, P. G., von Wolff, M., et al. (1999) Regulation of TNF-alpha mRNA expression in endometrial cells by TNF- alpha and by oestrogen withdrawal. *Mol Hum Reprod*, **5**, 1141-9

Tabibzadeh, S. and Sun, X. Z. (1992) Cytokine expression in human endometrium throughout the menstrual cycle. *Hum Reprod*, **7**, 1214-21

Tabibzadeh, S., Zupi, E., Babaknia, A., et al. (1995b) Site and menstrual cycle-dependent expression of proteins of the tumour necrosis factor (TNF) receptor family, and BCL-2 oncoprotein and phase- specific production of TNF alpha in human endometrium. *Hum Reprod*, **10**, 277-86

Takeda, K., Takeuchi, O., Tsujimura, T., et al. (1999) Limb and skin abnormalities in mice lacking IKKalpha. *Science*, **284**, 313-6

Tanaka, M., Fuentes, M. E., Yamaguchi, K., et al. (1999) Embryonic lethality, liver degeneration, and impaired NF-kappa B activation in IKK-beta-deficient mice. *Immunity*, **10**, 421-9

Telfer, J. F., Irvine, G. A., Kohnen, G., et al. (1997) Expression of endothelial and inducible nitric oxide synthase in non- pregnant and decidualized human endometrium. *Mol Hum Reprod*, **3**, 69-75

Terranova, P. F., Hunter, V. J., Roby, K. F., et al. (1995) Tumor necrosis factor-alpha in the female reproductive tract. *Proc Soc Exp Biol Med*, **209**, 325-42

Thienel, T., Winterhager, E. and Chwalisz, K. (2000) NFκB is Upregulated during Decidualization and Correlates with Differentiation Markers. *J Soc Gynecol Investig*, **7**, 603

Thompson, J. E., Phillips, R. J., Erdjument-Bromage, H., et al. (1995) I kappa B-beta regulates the persistent response in a biphasic activation of NF-kappa B. *Cell*, **80**, 573-82

Thompson, R. C. and Ohlsson, K. (1986) Isolation, properties, and complete amino acid sequence of human secretory leukocyte protease inhibitor, a potent inhibitor of leukocyte elastase. *Proc Natl Acad Sci U S A*, **83**, 6692-6696

Thomson, A. J., Telfer, J. F., Young, A., et al. (1999) Leukocytes infiltrate the myometrium during human parturition: further evidence that labour is an inflammatory process. *Hum Reprod*, **14**, 229-36

Tomee, J. F., Hiemstra, P. S., Heinzel-Wieland, R., et al. (1997) Antileukoprotease: an endogenous protein in the innate mucosal defense against fungi. *J Infect Dis*, **176**, 740-747

Tomee, J. F., Koeter, G. H., Hiemstra, P. S., et al. (1998) Secretory leukoprotease inhibitor: a native antimicrobial protein presenting a new therapeutic option? *Thorax*, **53**, 114-6

Treloar, A. E., Boynton, R. E., Behn, B. G., et al. (1967) Variation of the Human Menstrual Cycle through Reproductive Life. *International Journal of Fertility*, **12**, 77-126

Tschugguel, W., Schneeberger, C., Unfried, G., et al. (1998) Induction of inducible nitric oxide synthase expression in human secretory endometrium [published erratum appears in Hum Reprod 1998 May;13(5):1414]. *Hum Reprod*, **13**, 436-44

Tsukamoto, N., Kobayashi, N., Azuma, S., et al. (1999) Two differently regulated nuclear factor kappaB activation pathways triggered by the cytoplasmic tail of CD40. *Proc Natl Acad Sci U S A*, **96**, 1234-9

Tung, L., Mohamed, M. K., Hoeffler, J. P., et al. (1993) Antagonist-occupied human progesterone B-receptors activate transcription without binding to progesterone response elements and are dominantly inhibited by A-receptors [see comments] [published erratum appears in Mol Endocrinol 1993 Nov;7(11):1378]. *Mol Endocrinol*, **7**, 1256-65

Tuschil, A., Lam, C., Haslberger, A., et al. (1992) Interleukin-8 stimulates calcium transients and promotes epidermal cell proliferation. *J Invest Dermatol*, **99**, 294-8

Udagwa, T., Woodside, D. G. and McIntyre, B. W. (1996) Alpha 4 beta 1 (CD49d/CD29) integrin costimulation of human T cells enhances transcription factor and cytokine induction in the absence of altered sensitivity to anti-CD3 stimulation. *J Immunol*, **157**, 1965-72

Ueda, A., Okuda, K., Ohno, S., et al. (1994) NF-kappa B and Sp1 regulate transcription of the human monocyte chemoattractant protein-1 gene. *J Immunol*, **153**, 2052-63

Valore, E. V., Park, C. H., Quayle, A. J., et al. (1998) Human beta-defensin-1: an antimicrobial peptide of urogenital tissues. *J Clin Invest*, **101**, 1633-1642

van der Burg, B. and van der Saag, P. T. (1996) Nuclear factor-kappa-B/steroid hormone receptor interactions as a functional basis of anti-inflammatory action of steroids in reproductive organs. *Mol Hum Reprod*, **2**, 433-8

Vegeto, E., Shahbaz, M. M., Wen, D. X., et al. (1993) Human progesterone receptor A form is a cell- and promoter-specific repressor of human progesterone receptor B function. *Mol Endocrinol*, **7**, 1244-55

Vincenti, M. P., Coon, C. I. and Brinckerhoff, C. E. (1998) Nuclear factor kappaB/p50 activates an element in the distal matrix metalloproteinase 1 promoter in interleukin-1beta-stimulated synovial fibroblasts. *Arthritis Rheum*, **41**, 1987-94

Wagner, B. L., Norris, J. D., Knotts, T. A., et al. (1998) The nuclear corepressors NCoR and SMRT are key regulators of both ligand- and 8-bromo-cyclic AMP-dependent transcriptional activity of the human progesterone receptor. *Mol Cell Biol*, **18**, 1369-78

Walz, A., Peveri, P., Aschauer, H., et al. (1987) Purification and amino acid sequencing of NAF, a novel neutrophil- activating factor produced by monocytes. *Biochem Biophys Res Commun*, **149**, 755-61

Wang, H., Critchley, H. O., Kelly, R. W., et al. (1998) Progesterone receptor subtype B is differentially regulated in human endometrial stroma. *Mol Hum Reprod*, **4**, 407-12

Wegmann, T. G., Lin, H., Guilbert, L., et al. (1993) Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? *Immunol Today*, **14**, 353-6

Wen, D. X., Xu, Y. F., Mais, D. E., et al. (1994) The A and B isoforms of the human progesterone receptor operate through distinct signaling pathways within target cells. *Mol Cell Biol*, **14**, 8356-64

Wesche, H., Henzel, W. J., Shillinglaw, W., et al. (1997) MyD88: an adapter that recruits IRAK to the IL-1 receptor complex. *Immunity*, **7**, 837-47

Wiedow, O., Harder, J., Bartels, J., et al. (1998) Antileukoprotease in human skin: an antibiotic peptide constitutively produced by keratinocytes. *Biochem Biophys Res Commun*, **248**, 904-9

Wissink, S., van Heerde, E. C., vand der Burg, B., et al. (1998) A dual mechanism mediates repression of NF-kappaB activity by glucocorticoids. *Mol Endocrinol*, **12**, 355-63

Woronicz, J. D., Gao, X., Cao, Z., et al. (1997) IkappaB kinase-beta: NF-kappaB activation and complex formation with IkappaB kinase-alpha and NIK. *Science*, **278**, 866-9

Yamaoka, S., Courtois, G., Bessia, C., et al. (1998) Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell*, **93**, 1231-40

Yeaman, G. R., Collins, J. E., Currie, J. K., et al. (1998) IFN-gamma is produced by polymorphonuclear neutrophils in human uterine endometrium and by cultured peripheral blood polymorphonuclear neutrophils. *J Immunol*, **160**, 5145-53

Yin, M. J., Yamamoto, Y. and Gaynor, R. B. (1998) The anti-inflammatory agents aspirin and salicylate inhibit the activity of I(kappa)B kinase-beta [see comments]. *Nature*, **396**, 77-80

Yokoo, T. and Kitamura, M. (1996) Dual regulation of IL-1 beta-mediated matrix metalloproteinase-9 expression in mesangial cells by NF-kappa B and AP-1. *Am J Physiol*, **270**, F123-30

Yoshida, S., Ono, M., Shono, T., et al. (1997) Involvement of interleukin-8, vascular endothelial growth factor, and basic fibroblast growth factor in tumor necrosis factor alpha-dependent angiogenesis. *Mol Cell Biol*, **17**, 4015-23

Yoshiki, N., Kubota, T. and Aso, T. (2000) Expression and localization of inducible nitric oxide synthase in human non-pregnant and early pregnant endometrium. *Mol Hum Reprod*, **6**, 283-287

Yoshimura, T. and Leonard, E. J. (1990) Secretion by human fibroblasts of monocyte chemoattractant protein-1, the product of gene JE. *J Immunol*, **144**, 2377-83

Yoshimura, T., Matsushima, K., Oppenheim, J. J., et al. (1987) Neutrophil chemotactic factor produced by lipopolysaccharide (LPS)- stimulated human blood mononuclear leukocytes: partial characterization and separation from interleukin 1 (IL 1). *J Immunol*, **139**, 788-93

Yoshimura, T., Yuhki, N., Moore, S. K., et al. (1989) Human monocyte chemoattractant protein-1 (MCP-1). Full-length cDNA cloning, expression in mitogen-stimulated blood mononuclear leukocytes, and sequence similarity to mouse competence gene JE. *FEBS Lett*, **244**, 487-93

Yue, T. L., Wang, X., Sung, C. P., et al. (1994) Interleukin-8. A mitogen and chemoattractant for vascular smooth muscle cells. *Circ Res*, **75**, 1-7

Zabel, U. and Baeuerle, P. A. (1990) Purified human I kappa B can rapidly dissociate the complex of the NF- kappa B transcription factor with its cognate DNA. *Cell*, **61**, 255-65

Zandi, E., Chen, Y. and Karin, M. (1998) Direct phosphorylation of IkappaB by IKKalpha and IKKbeta: discrimination between free and NF-kappaB-bound substrate. *Science*, **281**, 1360-3

Zandi, E. and Karin, M. (1999) Bridging the gap: composition, regulation, and physiological function of the IkappaB kinase complex. *Mol Cell Biol*, **19**, 4547-51

Zandi, E., Rothwarf, D. M., Delhase, M., et al. (1997) The IkappaB kinase complex (IKK) contains two kinase subunits, IKKalpha and IKKbeta, necessary for IkappaB phosphorylation and NF-kappaB activation. *Cell*, **91**, 243-52

Zhang, J., Lathbury, L. J. and Salamonsen, L. A. (2000) Expression of the chemokine eotaxin and its receptor, CCR3, in human endometrium. *Biol Reprod*, **62**, 404-11

Zhang, Y., Cao, H. J., Graf, B., et al. (1998) CD40 engagement up-regulates cyclooxygenase-2 expression and prostaglandin E2 production in human lung fibroblasts. *J Immunol*, **160**, 1053-7

Zhang, Y., DeWitt, D. L., McNeely, T. B., et al. (1997) Secretory leukocyte protease inhibitor suppresses the production of monocyte prostaglandin H synthase-2, prostaglandin E2, and matrix metalloproteinases. *J Clin Invest*, **99**, 894-900

Zhao, Q. and Lee, F. S. (1999) Mitogen-activated protein kinase/ERK kinase kinases 2 and 3 activate nuclear factor-kappaB through IkappaB kinase-alpha and IkappaB kinase- beta. *J Biol Chem*, **274**, 8355-8

Zhou, J., Dsupin, B. A., Giudice, L. C. et al. (1994) Insulin-like growth factor system gene expression in human endometrium during the menstrual cycle. *J Clin Endocrinol Metab*, **79**, 1723-34

Appendix 1: General Materials

TISSUE COLLECTION	SOURCE
RPMI 1640 medium	Sigma, Poole, Dorset, UK
NBF	See Appendix 2
Hanks Balanced Salt Solution	Life Technologies Ltd., Paisley, UK
HEPES	Flow Laboratories, Irvine, UK
Phosphate buffered saline	Sigma
Sucrose	Sigma
OCT freezing compound	Tissue-Tek, Elkhart, USA

RNA EXTRACTION	SOURCE
Tri Reagent	Sigma
Ultraspec RNA Isolation System	Biogenesis Ltd., Poole, UK
chloroform	BDH Laboratory Supplies, Poole, UK
isopropanol	Sigma-Aldrich Co. Ltd.
ethanol	Hayman Ltd., Essex, UK
RNA storage solution	Ambion Inc, Austin, Texas, USA

RT-PCR	SOURCE
all reagents unless stated otherwise	PE Biosystems, Warrington, UK
mineral oil	Sigma

IMMUNOHISTOCHEMISTRY	SOURCE
neutral buffered formalin (NBF)	see Appendix 2
phosphate buffered saline (PBS)	see Appendix 2
PBS + Tween	see Appendix 2
hydrogen peroxide	BDH
horse/goat serum	Vector Laboratories, Peterborough, UK
0.01M sodium citrate	see Appendix 2
mouse/rabbit immunoglobulin	Vector Laboratories
goat immunoglobulin	R&D Systems, Oxford, UK
anti-NIK	Santa Cruz Biotechnologies Inc, California, USA
anti-IKKα	Santa Cruz Biotechnologies Inc
anti-CD40	Dr. Ed Clark, Seattle, Washington, USA
anti-Thy-1	Clone F15-421-5 (Smith, et al., 1995)
anti-CD1a	Immunotech, Marseille, France
anti-SLPI	R&D Systems

IMMUNOHISTOCHEMISTRY	SOURCE
anti-cytokeratin	Dako Ltd., Cambridge, UK
biotinylated horse α mouse/ goat α rabbit/ horse α goat	Vector Laboratories
avidin-biotin complex (ABC)	Elite ABC 6101, Vector Laboratories or Dako Ltd.
diaminobenzidine	Vector Laboratories or Dako Ltd
Harris's haematoxylin	Pioneer Research Chemicals Ltd, Colchester, UK
histoclear	National Diagnostics, Atlanta, Georgia, USA
pertex	Cellpath plc, Hemel Hempsted, UK
xylene	BDH

TISSUE & CELL CULTURE	SOURCE
polypropylene capillary matting	Garden Centre
RPMI 1640 medium	Sigma
fetal calf serum	Mycoplex, PAA Laboratories, Teddington, UK
penicillin	Sigma
streptomycin	Sigma
gentamycin	Sigma
24 well culture plates	Corning Costar, High Wycombe, UK
$25cm^2$ culture flasks	Corning Costar
Tris buffer	See Appendix 2
HCl	Sigma
NaOH	Sigma
dexamethasone	Sigma
epidermal growth factor (EGF)	Sigma
forskolin	Sigma
indomethacin	Sigma
Interferon γ (IFNγ)	Peprotech, London, UK
Interleukin-10 (IL-10)	Peprotech
Interleukin-12 (IL-12)	Peprotech
Interleukin-1β (IL-1β)	Peprotech

TISSUE & CELL CULTURE	SOURCE
Interleukin-6 (IL-6)	R&D Systems
lipopolysaccharide (LPS) – E. coli	Sigma
lipoteichoic acid (LTA)	Sigma
MG132	Biomol Research Laboratories Inc., Plymouth Meeting, PA, USA
oestradiol	Sigma
phorbol, 1-myristate, 13-acetate (PMA)	Calbiochem, Nottingham, UK
progesterone	Sigma
prolactin	Gift from Prof. A. McNeilly, Medical Research Council, Human Reproductive Sciences Unit, Edinburgh
prostaglandin E_2 (PGE_2)	Gift from Control Therapeutics, East Kilbride, UK
prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$)	Cayman Chemicals, Ann Arbor, MI, USA
rolipram	Sigma
SN50	Biomol
tumour growth factor α (TGFα)	Peprotech
tumour necrosis factor α (TNFα)	Sigma

ELISA	SOURCE
96 well assay plates	Nunc Maxi-Sorp, Gibco, Paisley, UK
recombinant SLPI	R&D Systems
anti-SLPI	R&D Systems
peroxidase labeled anti-sheep/goat IgG Fab fragments	Boehringer Mannheim, Lewes, UK
IL-8 standard	Toray Industries, Tokyo, Japan
anti-IL-8 detection antibody	R&D Systems
anti-IL-8 coating antibody	R&D Systems
carbonate buffer	see Appendix 2
PBS	Sigma
blocking/protecting solution	see Appendix 2
wash buffer	see Appendix 2
substrate	see Appendix 2
2N sulphuric acid	BDH

Appendix 2: Recipes for solutions

All chemicals listed were from Sigma and all dilutions were in distilled water unless otherwise stated.

1. Blocking/protecting solution

In 1 litre:	20g polyvinylpyrollidone 2%	
	5g BSA	
	1ml preservatives	Boehringer Mannheim
	1.9g EDTA	
	6.1g Tris	

2. Carbonate coating buffer

In 1 litre:	4.2g Na_2CO_3	BDH
	35.0g $NaHCO_3$	BDH
	preservatives	
	pH 9.6	

3. ELISA buffer

In 1 litre:	12.1g Tris	
	2g BSA	
	9g NaCl	
	0.7g EDTA	
	300µl phenol red	Flow Laboratories, UK
	1ml preservatives	
	pH 7.2	

4. ELISA substrate

1ml tetramethyl benzidine : 1ml urea hydrogen peroxide : 10ml sodium acetate

100mM sodium acetate

In 1 litre: 13.6g sodium trihydrate
1ml preservatives
pH6

tetramethyl benzidine

3mg/ml in dimethylformamide

urea hydrogen peroxide

0.5% in 50mM sodium acetate, pH6

5. ELISA wash buffer

In 1 litre: 5ml Tween 20
90g NaCl
12.1g Tris
pH 7-7.5
Dilute 1:20 to use

6. Neutral buffered formalin (NBF)

In 1 litre:	6.5g Na_2HPO_4	BDH
	4.5g $NaH_2PO_4.2H_20$	BDH
	100ml 40% formaldehyde	
	900ml distilled water	

7. Phosphate buffered saline (PBS)

In 1 litre:	5 PBS tablets
	pH 7.4-7.6

8. PBS + Tween

In 1 litre:	5 PBS tablets
	8g NaCl
	100µl Tween-20
	pH 7.4-7.6

9. 0.1M sodium citrate buffer

In 1litre:	29.4g Tri-sodium citrate	BDH
	0.1g sodium azide	
	pH 6	
	Diluted 1:10 to use	

10. Tris buffer

In 1 litre:	121.1g of Trizma base
	PH 7.2

Appendix 3: Conference Proceedings

King, A.E., Critchley, H.O.D., Denison, F.C. and Kelly, R.W. (1998) Decidualization of human endometrium is associated with release of secretory leukocyte protease inhibitor. 11th Simpson Symposium, Edinburgh. UK.

King, A.E., Critchley, H.O.D. and Kelly, R.W. (1998) Secretory leukocyte protease inhibitor is present in first trimester decidua. Journal of Endocrinology 159, Supplement, P56.

King, A.E., Phipps, R.P., Critchley, H.O.D. and Kelly, R.W. (1999) The distribution of CD40 in human endometrium: a mechanism for activation of inflammatory mediators associated with the vasculature? Oral presentation at 5th International Congress on The Cell Biology of Reproduction, Cambridge, UK.

King, A.E., Critchley, H.O.D. and Kelly, R.W. (2000) The NFκB pathway in human endometrium and first trimester decidua. Journal of Reproduction and Fertility. Abstract Series Number 25, 179. Fertility 2000. Edinburgh, UK.

Appendix 4: Publications

King, A.E., Critchley, H.O.D., and Kelly, R.W. (2000) Presence of secretory leukocyte protease inhibitor in human endometrium and first trimester decidua suggests an antibacterial protective role. Molecular Human Reproduction 6:2:191-196

King, A.E., Kelly, R.W., Critchley, H.O.D., Malmstrom, A., Sennstrom, M. and Phipps, R.P. CD40 Expression in Uterine Tissues: A Key Regulator of Cytokine Expression by Fibroblasts. Submitted to Journal of Clinical Endocrinology and Metabolism.

King, A.E., Critchley, H.O.D., and Kelly, R.W. The NFκB Pathway in Human Endometrium and First Trimester Decidua. Submitted to Molecular Human Reproduction.

Molecular Human Reproduction vol.6 no.2 pp. 191–196, 2000

Presence of secretory leukocyte protease inhibitor in human endometrium and first trimester decidua suggests an antibacterial protective role

Anne E.King[1,3], Hilary O.D.Critchley[2] and Rodney W.Kelly[1]

[1]Medical Research Council Reproductive Biology Unit and [2]Obstetrics and Gynaecology, University of Edinburgh, Centre for Reproductive Biology, 37 Chalmers Street, Edinburgh EH3 9ET, UK

[3]To whom correspondence should be addressed

Secretory leukocyte protease inhibitor (SLPI) is a neutrophil elastase inhibitor which also has antibacterial and anti-inflammatory properties. It is found associated with mucosal membranes. Although SLPI has been reported in the cervix it has not thus far been reported in human endometrium. This study investigates the presence of SLPI in endometrium, first trimester decidua and trophoblast. Cultured first trimester decidua was found to produce 4.7 ± 2.0 ng/mg/24 h of SLPI. Endometrium and trophoblast were both found to secrete significantly lower amounts of SLPI ($P < 0.01$) although endometrial expression was menstrual cycle dependent with increased secretion in the secretory phase. Although relatively low concentrations of SLPI were released from the endometrium during culture, most of the SLPI remained associated with the tissue and could be recovered with mild acid extraction. This is in agreement with the high isoelectric point (pI) for SLPI, associated with high solubility at low pH. The main site of SLPI synthesis in endometrium and decidua was found to be the glandular epithelium. An antibiotic role for SLPI in the endometrium and decidua during implantation and pregnancy would be consistent with the expression profile and localization of SLPI.

Key words: defensins/endometrium/epithelial glands/natural antibiotics/SLPI

Introduction

Secretory leukocyte protease inhibitor (SLPI) is a 11.7 kDa cysteine-rich protein found associated with mucosal surfaces, e.g. lung and cervix (Franken *et al.*, 1989). SLPI is produced by neutrophils (Bohm *et al.*, 1992), macrophages (Jin *et al.*, 1997) and epithelial cells (Abe *et al.*, 1991). SLPI is thought to function mainly as a serine protease inhibitor (serpin) and has been characterized as a critical inhibitor of neutrophil elastase in the lung (Thompson and Ohlsson, 1986). There is evidence to suggest that SLPI has a protective function at mucosal membranes. SLPI has been shown to kill both *Escherichia coli* and *Staphylococcus aureus*, thus showing bactericidal activity against gram-negative and gram-positive bacteria (Hiemstra *et al.*, 1996). Antiviral (McNeely *et al.*, 1995) and antifungal (Tomee *et al.*, 1997) effects have also been observed. Secondly, evidence suggests that SLPI acts to inhibit various proinflammatory systems. For example, in mouse macrophages SLPI inhibits the actions of lipopolysaccharide (LPS) (Jin *et al.*, 1997). Also in human monocytes, SLPI decreases the production of matrix metalloproteinases (responsible for extracellular matrix degradation) via suppression of a cyclo-oxygenase-2/prostaglandin E_2/cAMP pathway (Zhang *et al.*, 1997). Receptors for SLPI have been identified in human monocytes (McNeely *et al.*, 1997). Finally, SLPI inhibits activation of the NFκB signal transduction pathway (Jin *et al.*, 1997) which is a major inducer of proinflammatory genes. SLPI is thus a pleiotropic molecule protecting against inflammatory insult and infection in a variety of ways.

In a reproductive context, SLPI has been detected in human cervical mucosa (Casslen *et al.*, 1981), term decidua (Denison *et al.*, 1999) and seminal plasma (Franken *et al.*, 1989). It has also been localized to the glandular and luminal epithelial cells of maternal endometrium of pig (Reed *et al.*, 1996) and the mRNA has been detected in horse and cow endometrium during pregnancy (Badinga *et al.*, 1994). It has been suggested that SLPI acts to maintain the uterine–placental border in these species and that expression is related to epitheliochorial placentation. SLPI has not been detected in the endometrium of mammals with haemochorial placentation (e.g. rat) (Badinga *et al.*, 1994) and previous studies have failed to detect the protein in non-pregnant human endometrium (Casslen *et al.*, 1981; Franken *et al.*, 1989), although SLPI has been found to be present in uterine fluid (Casslen *et al.*, 1981). Successful human pregnancy demands that inflammatory responses in the uterus are suppressed and, as an antibacterial and anti-inflammatory molecule, it seems probable that SLPI may contribute to this. This study investigates the expression of SLPI by human endometrium, decidua and trophoblast.

Materials and methods

Tissue collection

Endometrial biopsies were collected from women undergoing gynaecological procedures for benign conditions. No attempt was made to distinguish between subjects with normal menstrual loss and undergoing a laparoscopic procedure for sterilization and subjects describing heavy menstrual loss and undergoing investigation or treatment (hysterectomy) for this subjective complaint. All women reported regular menstrual cycles (25–35 days) and had not received

any form of hormonal treatment in the 3 months preceding biopsy. Biopsies were dated from the patient's last menstrual period (LMP); histological dating according to published criteria (Noyes *et al.*, 1950) was consistent with the date of LMP. Furthermore, circulating sex steroid concentrations were in keeping with the time in the cycle during which the biopsy was collected. Serum was separated from venous blood samples collected at the time of biopsy. Oestradiol and progesterone concentrations were measured by radioimmunoassay. The inter-assay coefficients of variation for the oestradiol and progesterone assays were 11.0 and 9.0% respectively, while the intra-assay coefficients of variation were 8.0 and 7.0% respectively. Oestradiol concentrations were in the range 0–445 pmol/l in the proliferative phase; all progesterone concentrations were <6 nmol/l. In the secretory phase, oestradiol concentrations were in the range 65–740 pmol/l; progesterone concentrations were >6 nmol/l (with the exception of two premenstrual samples).

In addition, endometrium was collected from women using a levonorgestrel intrauterine system (LNG–IUS; $n = 4$) for contraception and heavy menses. The LNG–IUS causes marked decidualization of the endometrium (Critchley *et al.*, 1998).

First trimester decidua were collected by curettage of the uterine wall away from the site of implantation prior to suction termination of pregnancy. Trophoblastic villi were also collected during the procedure. Decidual parietalis (without trophoblast) was subsequently confirmed by examination of haematoxylin and eosin-stained sections.

Tissue samples were collected in Roswell Park Memorial Institute (RPMI) 1640 medium (Sigma, Poole, Dorset, UK); in addition, endometrial biopsies were fixed in 10% neutral-buffered formalin (NBF) overnight at 4°C, stored in 70% ethanol, and then embedded in wax. Written informed consent was received from all patients prior to biopsy collection and ethical approval was received from Lothian Research Ethics Committee.

Tissue culture

All tissue was cultured for 24 h on sterilized polypropylene capillary matting in RPMI 1640 (Sigma) supplemented with 10% fetal calf serum (Mycoplex, PAA Laboratories, Teddington, UK), penicillin (50 µg/ml; Sigma), streptomycin (50 µg/ml; Sigma) and gentamycin (5 µg/ml; Sigma). Endometrium and decidua were cultured in the presence of oestradiol (10^{-8} mol/l). The culture medium was removed after 24 h for subsequent inclusion in SLPI assays. Tissue was weighed after incubation and all assay measurements were corrected for weight.

Acid extraction of tissue

SLPI is an acid-stable molecule with a high isoelectric (pI) point and, therefore, more soluble in weak acid solution. After 24 h culture, tissue was acid extracted in the presence of 200 µl phosphate-buffered saline (PBS) and 10 µl HCl (1 N) for 10 min. Neutralization was achieved by addition of 10 µl NaOH (1 N) and 50 µl Tris buffer (1 mol/l; pH 7.2). Supernatant was collected for subsequent inclusion in SLPI assays.

SLPI assay

SLPI was measured by an enzyme-linked immunosorbent assay (ELISA). Assay plates (96-well; Nunc Maxi-Sorp, Gibco, Paisley, UK) were coated with 0.025 µg/ml recombinant SLPI (R&D Systems, Oxford, UK) in PBS and 1% 400 mmol/l carbonate buffer. 100 µl were added to each well. Plates were left for 60 min at room temperature. Blocking was carried out with 400 µl/well blocking/protecting solution [polyvinylpyrrolidone 2%, bovine serum albumin (BSA) 5 mg/ml, preservatives, EDTA 5 mmol/l, Tris 50 mmol/l] for 30 min. Plates were washed with wash buffer (150 mmol/l NaCl, 100 mmol/l Tris, 0.05% Tween-20; pH 7–7.5). 150 µl of standard/sample and 50 µl of anti-SLPI (R&D Systems); 2 µg/ml; diluted in ELISA buffer (150 mmol/l NaCl, 100 mmol/l Tris, 50 mmol/l Phenol Red solution, 2 mmol/l EDTA, 1 mmol/l 2-methylisothiazolone) (Boehringer Mannheim, Lewes, UK), 1 mmol/l bromonitrodioxane (Boehringer Mannheim), 2 mg/ml BSA, 0.05% Tween-20; (pH 7.2) were added to each well. A non-specific binding well (200 µl buffer only) and two B_0 wells (150 µl buffer; 50 µl anti-SLPI) were included on each plate. Standards were added in triplicate and their concentration range was from 50–0.098 ng/ml. Incubation was on a plate shaker at room temperature for 2 h; and the plates were then washed. 100 µl/well of peroxidase-labelled anti-sheep/goat immunoglobulin G (IgG) Fab fragments raised in donkey (Boehringer Mannheim; 1:1000 dilution of stock in ELISA buffer) were added. Incubation was on a shaker as before and the plates were washed again. 200 µl substrate (0.3 g/l urea–hydrogen peroxide, 0.1 g/l tetramethyl benzidine in 100 mmol/l sodium acetate; pH 6) were added to each well. After 2–10 min, wells were quenched with 50 µl/well 2 N sulphuric acid. Plates were read in a plate reader at 450 nm.

Immunohistochemistry

Tissue sections were dewaxed in histoclear (National Diagnostics, Atlanta, Georgia, USA) and rehydrated in descending grades of alcohol. Non-specific endogenous peroxidase activity was blocked by treatment with 3% hydrogen peroxide (BDH Laboratory Supplies, Poole, UK) in distilled water for 10 min at room temperature. All tissue sections were subjected to a non-immune block with diluted normal horse serum (Vectastain 4002; Vector Laboratories, Peterborough, UK) for 20 min in a humidified chamber at room temperature. Tissue sections were then incubated overnight at 4°C with 50 µl of goat anti-SLPI antibody (1:400 in horse serum; R&D Systems). In negative control sections the primary antibody was substituted with goat immunoglobulin (R&D Systems). Sections were then incubated with biotinylated horse-anti goat IgG (Vector Laboratories) followed by an avidin–biotin peroxidase detection system (both for 60 min at room temperature; Elite ABC 6101; Vector Laboratories). The peroxidase substrate diaminobenzidine (DAB; Vector Laboratories) was used to identify positive staining. Sections were then counterstained with Harris's haemotoxylin (Pioneer Research Chemicals Ltd, Colchester, UK), dehydrated in ascending grades of alcohol and mounted from xylene in Pertex (Cellpath plc, Hemel Hempsted, UK).

SLPI reverse transcription–polymerase chain reaction (RT–PCR)

Tissue samples were immersed in Ultraspec (Ultraspec RNA Isolation System, Biogenesis Ltd, Poole, UK), homogenized and RNA was extracted as detailed in the manufacturer's protocol.

Decidual and endometrial RNA samples were reverse transcribed using random primers with $MgCl_2$ (5.5 mmol/l), dNTPs, random hexamers (2.5 µmol/l), RNAase inhibitor (0.4 IU/µl) and multiscribe reverse transcriptase (1.25 IU/µl; all from PE Biosystems, Warrington, UK). The mix was divided into aliquots in individual tubes (8 µl/tube) and template RNA was added (2 µl/tube of 100 ng/µl RNA). Mineral oil was added and samples were incubated for 60 min at 25°C, 45 min at 48°C and then at 95°C for 5 min.

A reaction mix was made containing Taqman buffer, $MgCl_2$ (5.5 mmol/l), dATP (200 µmol/l), dCTP (200 µ mol/l), dGTP (200 mmol/l), dUTP (400 µmol/l), ribosomal 18S forward and reverse primers and probe (all at 50 nmol/l), SLPI forward and reverse primers (both 300 nmol/l), SLPI probe (200 nmol/l), AmpErase UNG (0.01 IU/µl) and Amplitaq Gold DNA polymerase (0.025 IU/µl; all from PE Biosystems). The mixture was divided into aliquots in

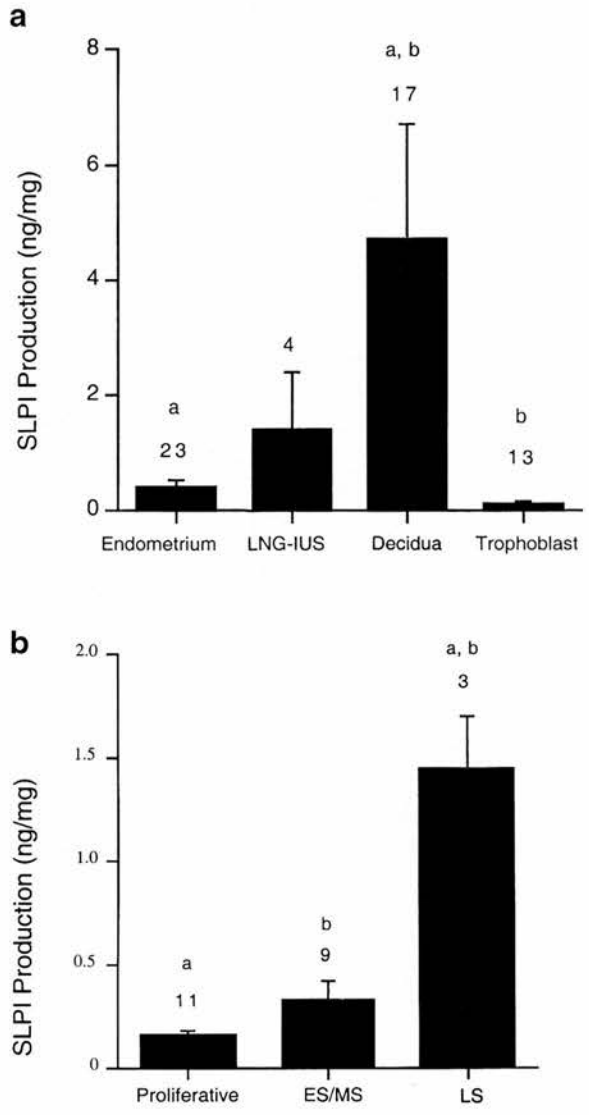


Figure 1. (**a**) Secretory leukocyte protease inhibitor (SLPI) production by control endometrium (throughout cycle), endometrium from levonorgestrel–intrauterine system users (LNG–IUS), decidua and trophoblast ($^{a,b}P < 0.01$). Numbers in the groups are shown immediately above the error bars. (**b**) SLPI production by control endometrium from the proliferative, early/mid-secretory (ES/MS) and late secretory (LS) phases of the menstrual cycle ($^{a,b}P < 0.0001$).

separate tubes for each cDNA sample. 1 µl/replicate of cDNA was added to each tube. After mixing 23 µl of sample was added to the wells on a PCR plate. Each sample was added in triplicate. A no template control (containing water) was included in triplicate. Wells were sealed with optical caps and the PCR reaction run on ABI Prism 7700 using standard conditions.

SLPI primers and probe for quantitative PCR were designed using the PRIMER express program (PE Biosystems). The sequence of the SLPI primers and probe were forward: GCATCAAATGCCTGGATCCT; reverse: GCATCAAACATTGGCCATAAGTC; probe (Fam labelled): TGACACCCCAAACCCAACAAGGAGG.

Ribosomal 18S primers and probe were forward: CGGCTACCACATCCAAGGAA; reverse: GCTGGAATTACCGCGGCT; probe (Joe labelled): TGCTGGCACCAGACTTGCCCTC.

The concentrations of SLPI primers and probe used in the PCR reaction were optimized and then validated using serial dilution of a

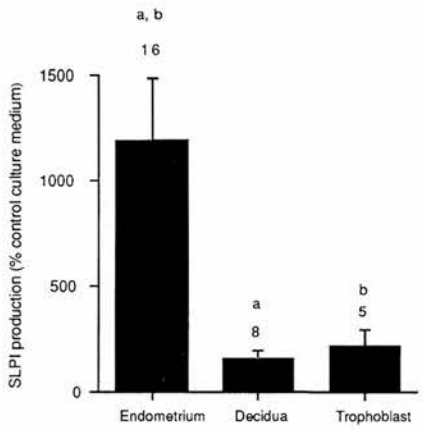


Figure 2. Secretory leukocyte protease inhibitor (SLPI) release during acid extraction of control endometrium, decidua and trophoblast ($^{a,b}P < 0.05$).

standard pool of RNA. Within assay variation of the PCR measurement of SLPI in cDNA was calculated from six replicates.

A PCR using the above primers (but excluding the probe) was run with identical conditions to the quantitative run. The product was run on an agarose gel to demonstrate amplification of a single product.

Statistical analysis

Significant difference was determined by analysis of variance (ANOVA) (Statview 3.0) and individual differences were assigned using Fisher's protected least squares differences (PLSD) test.

Results

SLPI secretion by endometrium, decidua and trophoblast

Decidua ($n = 17$) produces 4.7 ± 2.0 ng/mg of SLPI. This is significantly higher ($P < 0.01$) than secretion by either endometrium or trophoblast which produce 0.4 ± 0.1 ng/mg and 0.1 ± 0.04 ng/mg respectively. Endometrium from LNG–IUS users produces 1.4 ± 1.0 ng/mg. This is similar to production by control endometrium (Figure 1a).

SLPI secretion by endometrium increases in the late secretory phase of the menstrual cycle. Secretion is 1.5 ± 0.2ng/mg at this time, compared with 0.2 ± 0.02ng/mg in the proliferative phase and 0.3 ± 0.1ng/mg in the early/mid-secretory phase (Figure 1b; late secretory compared with proliferative and early/mid-secretory, $P < 0.0001$).

SLPI release from endometrium, decidua and trophoblast during acid extraction

Endometrium releases 1185 ± 298% (of SLPI in control culture medium) during acid extraction whereas decidua releases only 150 ± 43%. Release from trophoblast (210 ± 72%) is also lower than from endometrium. (Figure 2; $P < 0.05$). This suggests that the majority of SLPI produced by endometrium is not secreted into the culture medium. The result from one decidual sample was discounted from this analysis as the value was 11805% (i.e. 94 SD from the mean of the other values).

Localization of SLPI in endometrium and decidua

Immunohistochemistry showed positive SLPI immunoreactivity in the glandular epithelium and secretions in endo-

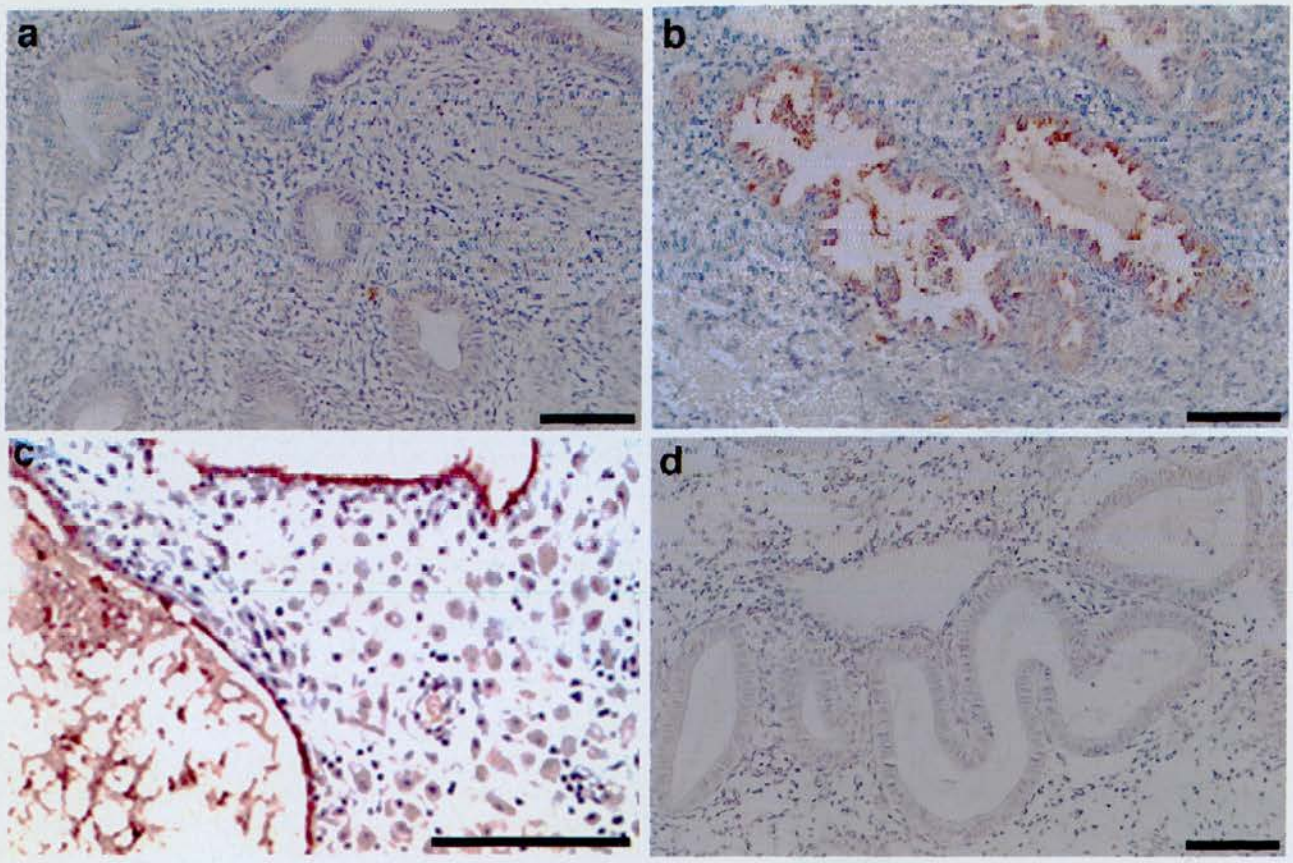


Figure 3. Immunohistochemical localization of secretory leukocyte protease inhibitor (SLPI) in human endometrium and decidua. (**a**) Proliferative endometrium. No immunoreactivity is present. (**b**) Mid-secretory endometrium. Immunoreactivity is present in the epithelial cells and secretions of the glands. No immunostaining is observed in the stroma. (**c**) First trimester decidua. SLPI immunoreactivity is present in the glandular epithelium and secretions. Immunostaining is present in stromal cells. (**d**) Negative control (primary antibody replaced with goat immunoglobulin at equimolar concentrations). Scale bars = 100μm.

metrial biopsies from the mid to late secretory phase. Immunostaining was also present in the luminal epithelium of some biopsies. The immunoreactivity was mainly localized to superficial areas of endometrium. No stromal staining occurred (Figure 3b). Very little SLPI was detected in proliferative and early secretory phase biopsies (Figure 3a).

SLPI immunostaining was also present in the epithelial cells of the glands and their secretions in decidual biopsies. Immunoreactivity was also found in the stromal cells of some decidua (Figure 3c).

SLPI RT–PCR

The accuracy of the quantitative PCR was tested by serial dilution (to 64× dilution) of a pool of *SLPI* cDNA. The slope of the line plotting the cycle number at which the curve crossed a threshold (Ct) against dilution had a gradient $<0.1\Delta$Ct units/64× dilution. Intra-assay precision of the PCR was calculated as 9.7% (r.s.d). All data were obtained from a single PCR run and related to a standard *SLPI* cDNA preparation using the formula $2^{-\Delta\Delta Ct}$ which relates the ratio of 18S and specific amplicon in the sample cDNA with that of the standard preparation.

SLPI mRNA was detected in all samples tested. Decidua contained 0.6 ± 0.3 of *SLPI* mRNA. This was greater than both proliferative and secretory endometrium which had 0.2 ± 0.1 and 0.5 ± 0.2 respectively (Figure 4a; not significant). Figure 4b shows that only one PCR product (representing SLPI) is amplified by the SLPI primers.

Discussion

To our knowledge this is the first report describing detection of SLPI in human endometrium and first trimester decidua. Previously, immunohistochemistry has failed to detect SLPI in human endometrium in either the proliferative or secretory phase of the menstrual cycle. However, SLPI was found to be present in uterine fluid and this was thought to be due to diffusion from the cervix (Casslen *et al.*, 1981). It now seems likely that the source of this SLPI was endometrial glandular epithelial cells. This is consistent with the production of SLPI by epithelial cells at other mucosal surfaces such as lung. SLPI has previously been detected in term decidua where it was found to be localized to the large decidualized stromal cells (Denison *et al.*, 1999). In first trimester decidua, SLPI was expressed mainly in the glandular compartment of the tissue although some stromal cells did contain SLPI. This suggests that as pregnancy progresses the site of SLPI production changes from the glandular epithelium to the stromal cells. A similar change of location has been reported in the case of the endothelin B (ET_B) receptor. This receptor is found

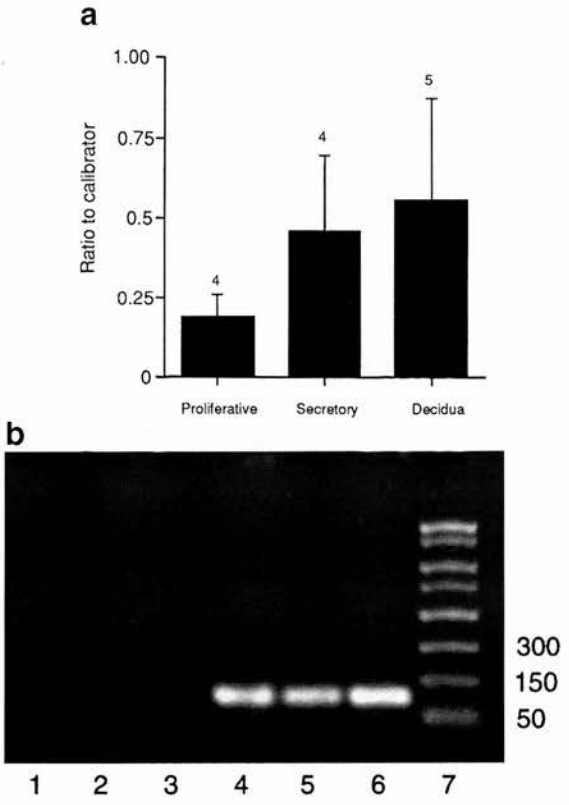


Figure 4. (**a**) Amounts of secretory leukocyte protease inhibitor (*SLPI*) mRNA in decidua and proliferative and secretory endometrium. (**b**) Agarose gel of polymerase chain reaction (PCR) products showing only one amplification product. Lanes 1–3 show no template controls. Lanes 4–6 show replicate samples from one patient containing SLPI. The product corresponds to the product length expected for SLPI (~110 bp). Lane 7 contains PCR markers.

in the glandular epithelium in secretory endometrium but at the time of menstruation and in decidualized tissue the receptor is also present in stromal cells (Kohnen *et al.*, 1998). It may be that as glands become atrophied as decidualization progesses it is necessary for the stromal cells to produce glandular products in order to maintain decidual function. Trophoblast was found to secrete very little SLPI and is unlikely to be a major source during pregnancy.

In non-pregnant endometrium, SLPI secretion shows cycle dependence with an increase in the mid to late secretory phase. This suggests progesterone regulation of SLPI although it is likely that this effect is indirect rather than a direct action of progesterone on *SLPI* gene expression. For example, changes to glandular morphology (e.g. increased surface area) occur and there are variations in inflammatory mediator expression under the influence of progesterone. Such effects may lead to secondary changes in endometrium, e.g. increased SLPI production. SLPI expression increases particularly in the late secretory phase around the time of decidualization and implantation and then increases further in decidua. The amounts of mRNA present are consistent with the protein expression pattern found. Leukocytes infiltrate the endometrium prior to menstruation and may be responsible for the rise in SLPI in the late secretory phase. However, this would be inconsistent with the increased presence of SLPI in decidua. Additionally, immunostaining suggested that the main source of SLPI

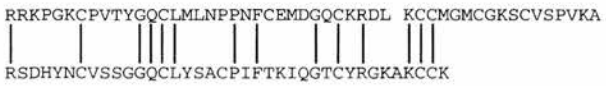


Figure 5. Comparison of secretory leukocyte protease inhibitor (SLPI) (upper sequence) and human β-defensin 1 (lower sequence) amino acid sequence.

in endometrium is the glands. Decidualization may be a contributory factor in SLPI production. LNG–IUS users have endometrium which is decidualized due to the very high local concentrations of progestogen (Pekonen *et al.*, 1992). Levonorgestrol-treated endometrium was found to produce higher concentrations of SLPI than proliferative endometrium but similar to those of late secretory endometrium.

Acid extraction of tissue was performed to ensure that the SLPI values measured in culture medium were a true representation of SLPI production by the tissue. Acid extraction of tissue resulted in release of SLPI from endometrium at concentrations much greater than those found during culture. Decidua and trophoblast released similar amounts of SLPI during culture and acid extraction. This suggests that SLPI production by endometrium far exceeds secretion although this may reflect in-vitro culture conditions. SLPI has been found to bind to endometrial extracellular matrix (Reed *et al.*, 1997) and it may be that acid extraction is causing release of bound protein.

As a potent neutrophil elastase inhibitor, SLPI may have a role in the prevention of tissue degradation in the endometrium. Neutrophils infiltrate the tissue during menstruation (Poropatich *et al.*, 1987) and may contribute to tissue breakdown. SLPI could act to limit this although is seems unlikely due to: (i) the superficial localization of the mediator; and (ii) the increased release of SLPI in pregnant decidua.

It has been suggested that in mammals which have epitheliochorial placentation, SLPI acts in maternal endometrium to maintain the integrity of the uterine–placental border by preventing inappropriate trophoblast invasion (Badinga *et al.*, 1994). In humans, trophoblast cells invade throughout the decidua and into the myometrium, so SLPI is unlikely to regulate this invasion because of its localization primarily in the superficial endometrium.

SLPI has been found to have antibacterial, antiviral and antifungal effects and it has previously been suggested that its presence in the cervix and the male genital tract (with high concentrations in semen) protects these mucosal membranes from insult (Ohlsson *et al.*, 1995). Endometrial production of SLPI could offer similar protection against infection of the uterus around the time of implantation and during pregnancy. Ascending infection is a major cause of premature labour, so natural antibiotics are likely to be important in controlling this. The location and expression profile of SLPI is consistent with an antibacterial role during pregnancy.

The defensins are a group of proteins found to have antibacterial, antifungal and antiviral actions. Defensin 5 and β-defensin 1 have been identified in epithelial cells of the endometrium and endocervix (Quayle *et al.*, 1998; Valore *et al.*, 1998). In addition, defensin 5 expression was found to be maximal in endometrium during the secretory phase of the

cycle (Quayle *et al.*, 1998). One area of the SLPI molecule has 37% homology with the defensins and five or six cysteine residues involved in disuphide bonding are conserved between the proteins (Figure 5). Interestingly this area of homology does not coincide with the *N*-terminus of the SLPI molecule which is thought to be involved in antibacterial activity (Hiemstra *et al.*, 1996). The similarities in protein sequence and expression of SLPI and the defensins gives further support to an antibacterial role for SLPI in endometrium.

Some bacteria secrete proteins which degrade SLPI implying that without this breakdown SLPI would pose a risk to the pathogen. *Trichomonas vaginalis*, a major pathogen of the lower female genital tract, releases cysteine proteases which degrade recombinant SLPI under assay conditions (Draper *et al.*, 1998). It has been reported that there may be an association between irregular menstrual bleeding and the presence of bacteria in the uterus (Kristiansen *et al.*, 1987; Moller *et al.*, 1995). Our study does not attempt to distinguish between patients with normal and heavy menstrual loss. Indeed, all patients included in the present study reported regular menstrual cycles. Further study is required to determine concentrations of SLPI in endometrium during uterine infection or in the context of aberrant menstrual bleeding patterns.

In addition to the antibacterial effects, SLPI also inhibits the NFκB signal transduction pathway (Jin *et al.*, 1997). This is a major pathway involved in inflammatory response. SLPI may have anti-inflammatory actions in decidua via inhibition of NFκB. This would prevent inappropriate inflammatory mediator expression during pregnancy.

In summary, SLPI has been detected for the first time in human non-pregnant endometrium and first trimester decidua. The most likely role of SLPI in this location is as a natural antibiotic and anti-inflammatory molecule. Infection ascending through the cervix could pose a threat to the implanting and developing conceptus. Indeed, the presence of high concentrations of SLPI in decidua might be some compensation for the immunosuppressive factors present in decidua of pregnancy.

Acknowledgements

We wish to acknowledge the help of Miss Teresa Drudy with the immunohistochemistry. We are grateful to Dr A.Williams for expert histological assessment of the endometrial biopsies.

References

Abe, T., Kobayashi, N., Yoshimura, K. *et al.* (1991) Expression of the secretory leukocyte inhibitor gene in epithelial cells. *J. Clin. Invest.*, **87**, 2207–2215.

Badinga, L., Michel, F.J., Fields, M.J. *et al.* (1994) Pregnancy-associated endometrial expression of antileukoproteinase gene is correlated with epitheliochorial placentation. *Mol. Reprod. Dev.*, **38**, 357–363.

Bohm, B., Aigner, T., Kinne, R. *et al.* (1992) The serine-protease inhibitor of cartilage matrix is not a chondrocytic gene product. *Eur. J. Biochem.*, **207**, 773–779.

Casslen, B., Rosengren, M. and Ohlsson, K. (1981) Localization and quantitation of a low molecular weight proteinase inhibitor, antileukoprotease, in the human uterus. Hoppe Seyler's *Z. Physiol. Chem.*, **362**, 953–961.

Critchley, H.O.D., Wang, H., Jones, R.L. *et al.* (1998) Morphological and functional features of endometrial decidualization following long-term intrauterine levonorgestrel delivery. *Hum. Reprod.*, **13**, 1218–1224.

Denison, F.C., Kelly, R.W., Calder, A.A. *et al.* (1999) Secretory leukocyte protease inhibitor concentration increases in amniotic fluid with the onset of labour in women: characterization of sites of release within the uterus. *J. Endocrinol.*, **161**, 299–306.

Draper, D., Donohoe, W., Mortimer, L. *et al.* (1998) Cysteine proteases of Trichomonas vaginalis degrade secretory leukocyte protease inhibitor. *J. Infect. Dis.*, **178**, 815–819.

Franken, C., Meijer, C.J.L.M. and Dijkman, J.H. (1989) Tissue distribution of antileukoprotease and lysozyme in humans. *J. Histochem. Cytochem.*, **37**, 493–498.

Hiemstra, P.S., Maassen, R.J., Stolk, J. *et al.* (1996) Antibacterial activity of antileukoprotease. *Infect. Immun.*, **64**, 4520–4524.

Jin, F.Y., Nathan, C., Radzioch, D. *et al.* (1997) Secretory leukocyte protease inhibitor: a macrophage product induced by and antagonistic to bacterial lipopolysaccharide. *Cell*, **88**, 417–426.

Kohnen, G., Campbell, S., Irvine, G.A. *et al.* (1998) Endothelin receptor expression in human decidua. *Mol. Hum. Reprod.*, **4**, 185–193.

Kristiansen, F.V., Oster, S., Frost, L. *et al.* (1987) Isolation of Gardnerella vaginalis in pure culture from the uterine cavity of patients with irregular bleedings. *Br. J. Obstet. Gynaecol.*, **94**, 979–984.

McNeely, T.B., Dealy, M., Dripps, D. J. *et al.* (1995) Secretory leukocyte protease inhibitor: a human saliva protein exhibiting anti-human immunodeficiency virus 1 activity in vitro. *J. Clin. Invest.*, **96**, 456–464.

McNeely, T.B., Shugars, D. C., Rosendahl, M. *et al.* (1997) Inhibition of human immunodeficiency virus type 1 infectivity by secretory leukocyte protease inhibitor occurs prior to viral reverse transcription. *Blood*, **90**, 1141–1149.

Moller, B.R., Kristiansen, F.V., Thorsen, P. *et al.* (1995) Sterility of the uterine cavity. *Acta Obstet. Gynecol. Scand.*, **74**, 216–219.

Noyes, R.W., Hertig, A.T. and Rock, J. (1950) Dating the endometrial biopsy. *Fertil. Steril.*, **1**, 3–25.

Ohlsson, K., Bjartell, A. and Lilja, H. (1995) Secretory leukocyte protease inhibitor in the male genital-tract–PSA-induced proteolytic processing in human semen and tissue localization. *J. Androl.*, **16**, 64–74.

Pekonen, F., Nyman, T., Lahteenmaki, P. *et al.* (1992) Intrauterine progestin induces continuous insulin-like growth factor- binding protein-1 production in the human endometrium. *J. Clin. Endocrinol. Metab.*, **75**, 660–664.

Poropatich, C., Rojas, M. and Silverberg, S.G. (1987) Polymorphonuclear leukocytes in the endometrium during the normal menstrual cycle. *Int. J. Gynecol. Pathol.*, **6**, 230–234.

Quayle, A.J., Porter, E.M., Nussbaum, A.A. *et al.* (1998) Gene expression, immunolocalization, and secretion of human defensin-5 in human female reproductive tract. *Am. J. Pathol.*, **152**, 1247–1258.

Reed, K.L., Badinga, L., Davis, D.L. *et al.* (1996) Porcine endometrial glandular epithelial cells in vitro: transcriptional activities of the pregnancy-associated genes encoding antileukoproteinase and uteroferrin. *Biol. Reprod.*, **55**, 469–477.

Reed, K.L., Michel, F.J., Hiemstra, P.S. *et al.* (1997) Interaction of secretory leukocyte protease inhibitor with placental and endometrial extracellular matrix. *Biol. Reprod.*, **56**, 489.

Thompson, R.C. and Ohlsson, K. (1986) Isolation, properties, and complete amino acid sequence of human secretory leukocyte protease inhibitor, a potent inhibitor of leukocyte elastase. *Proc. Natl. Acad. Sci. USA*, **83**, 6692–6696.

Tomee, J.F., Hiemstra, P.S., Heinzel-Wieland, R. *et al.* (1997) Antileukoprotease: an endogenous protein in the innate mucosal defense against fungi. *J. Infect. Dis.*, **176**, 740–747.

Valore, E.V., Park, C.H., Quayle, A.J. *et al.* (1998) Human b-defensin-1: an antimicrobicidal peptide of urogenital tissues. *J. Clin. Invest.*, **101**, 1633–1642.

Zhang, Y.H., DeWitt, D.L., McNeely, T.B. *et al.* (1997) Secretory leukocyte protease inhibitor suppresses the production of monocyte prostaglandin H synthase-2, prostaglandin E(2), and matrix metalloproteinases. *J. Clin. Invest.*, **99**, 894–900.

Received on May 28, 1999; accepted on October 21, 1999